MEASUREMENT OF 02 SATURATION IN FREELY DEEP DIVING NORTHERN ELEPHANT SEALS (*MIROUNGA ANGUSTIROSTRIS*) USING A NOVEL DATA LOGGING TAG

A Thesis

by

MELISSA A. BREWER

Submitted to the Office of Graduate and Professional Studies of Texas A&M University
and the Graduate Faculty of The Texas A&M University – Corpus Christi
in partial fulfillment of the requirements for the joint degree of

MASTER OF SCIENCE

Chair of Committee,	Andreas Fahlman
Committee Members,	Manuela Gardner
	Randall Davis
Head of Department,	Joe Fox

August 2015

Major Subject: Marine Biology

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ABSTRACT

MEASUREMENT OF 02 SATURATION IN FREELY DEEP DIVING NORTHERN ELEPHANT SEALS (*MIROUNGA ANGUSTIROSTRIS*) USING A NOVEL DATA LOGGING TAG

Melissa A. Brewer, B.S. Biology and B.S. Zoology, North Carolina State University

Chair of Advisory Committee: Dr. Andreas Fahlman

Marine mammals live a life of dual constraints and must balance energetic demands (oxygen consumption) and limited O_2 availability while breath-holding. In order to overcome these dual constraints, marine mammals have developed unique physiological traits to cope with their aquatic environment, thereby taking advantage of a unique foraging niche. Extending breath-hold dive time increases the opportunity to obtain nutrients, but it can be energetically costly if marine mammals exceed the threshold for aerobic metabolism. We know relatively little about the physiological mechanisms employed by these animals that allow them to flourish in such an extreme environment.

Due to the difficulty obtaining direct physiological measurements, there are gaps in knowledge for the field of diving physiology, particularly in the understanding of muscle perfusion during a dive. In order to improve our understanding of how marine mammals adjust muscle blood flow (oxygen and nutrients) during diving to increase dive time, we developed and tested a tag with oxygen sensors that attempted to measure O_2

saturation in the muscle of freely deep-diving elephant seals. The purpose of this part of the study aimed at using an archival tag and implanted sensor system to measure physiological variables in freely deep-diving Northern elephant seals.

Following the sensor and data logger development, we set out to test a long-held assumption that oximeter use in marine mammals could be calibrated with terrestrial mammal blood. This was of particular importance to this study as calibrations of the oximeter data loggers in the Northern elephant seal were needed. As part of the ability of marine mammals to prolong apnea for diving, some have modified hemoglobin to change its affinity for oxygen. These polymorphisms alter the affinity for O_2, possibly by altering the folding of the protein. Alteration in the quaternary structure may also alter the optical properties of Hb. Oximeters use the change in the optical properties of oxygenated (HbO_2) and reduced Hb (HbR) to determine arterial blood O_2 saturation. Given the differences in Hb isoformss, the optical properties need to be assessed before conventional veterinary oximeters can be used on marine species. Therefore, the objective of this part of the study was to determine the absorbance spectra of HbO_2 and HbR in several species of marine mammals (killer whale, short-finned pilot whale, beluga whale, and northern elephant seals) and compare these against humans. Whole blood samples were opportunistically obtained during routine health assessment, and the Hb was isolated via a series of centrifugation, dialysis, and filtration steps. The isolated Hb was oxygenated or deoxygenated using 5% CO_2 in 95% O_2 or N_2, respectively. Under gas-tight conditions, the HbO_2 and HbR samples were placed in a UV-visible light spectrophotometer and the absorption spectra measured from 600 nm to 1000 nm. The

absorption spectra were overlaid and compared between species. The results indicate the absorption spectra are similar between humans and the species investigated and the point where absorbance is equal for HbO_2 and HbR in all species at ~800 nm.

The results of the Hb study allowed the possibility of properly calibrating the oximeter we surgically implanted in the Northern elephant seal. The oximeter sensor contained 3 LEDs in the visible red, near infrared (NIR), and infrared (IR) spectral regions (emitting at red:660, NIR:810, and IR:940 nm, respectively) and a photo detector. The sensor was attached to the external data logger with a thin (2-3 mm diameter) flexible silver (Ag) cable. The external data logger was attached with epoxy to the skin and fur of the seal following implantation of the sensor.

In April 2013, 5 northern elephant seals were captured from the Año Nuevo and were instrumented with the oxygen sensor and data logger via aseptic surgical technique. Following implantation of the muscle O_2 sensor and attachment of logger and satellite and radio transmitters, the animals were allowed to recover. Every 1-2 hours signs of inflammation, infection, or changes in swimming behavior were documented and none were observed in any seal. After translocation, all of the seals returned to the Año Nuevo rookery. All 5 seals implanted returned to Año Nuevo, had the instrumentation easily and successfully removed, and showed no signs of infection, inflammation or trauma concluding that we were able to develop a surgical technique that minimized the invasiveness of implanting a small sensor into the major swimming muscle of the Northern elephant seal. Free-diving ocean data was collected from the oximeter data logger only from the last two seals implanted, however only 1 out of 5 had oximetry data.

Additionally, there was difficulty in calibrating the oximeter/sensor data logger. However, GPS tracks and dive patterns were recorded and analyzed from all 5 seals. All seals were equipped with GPS tags with ARGOS satellite uplink and had similar dive patterns to one another and to previous studies. Furthermore, we were able to obtain data by the end of the field season from one seal out of five indicating that with a few adjustments to the data logger, a second field season may yield a larger sample size and more reliable data.

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INTRODUCTION

While air-breathing terrestrial animals have an unlimited supply of oxygen to maintain aerobic metabolism, the supply for marine mammals is finite while foraging underwater. The physiological traits marine mammals possess to dive longer and deeper allow them to take advantage of a unique niche when foraging for food. Longer dive times increase the opportunity to provide food for themselves and their offspring while also decreasing exposure to predators, thereby increasing the likelihood of survival (Costa et al., 2001). However, we know relatively little about the physiological mechanisms employed by these animals that allow them to flourish in such an extreme environment.

The energetic cost of foraging is one of the most important considerations in understanding the limits of diving mammals. Marine mammals live a life of dual constraints and must balance energetic demands (oxygen consumption) and limited O_2 availability while breath-holding. In order to overcome these dual constraints, marine mammals have developed unique physiological traits to cope with their aquatic environment. Successful foraging is directly affected by prey location in the water column and the swimming effort and speed exerted to catch prey (Hassrick et al., 2007). Extending breath-hold dive time increases the opportunity to obtain nutrients, but it can be energetically costly if marine mammals exceed the threshold for aerobic metabolism, which requires more time at the surface to recover. Understanding the management of metabolic gases and the physiological traits that allow prolonged breath-hold dives are considered the “holy grail” of diving physiology (Mottishaw et al., 1999).

Aerobic dive limit

The most efficient strategy to convert biochemical nutrients to energy is aerobic respiration. However, when the cardio-respiratory system cannot meet the delivery demand of O_2, such as during exercise or breath-holding, the body switches to anaerobic metabolism in order to fuel the cells. As a by-product of anaerobic metabolism, lactate forms and accumulates in muscle tissue until oxygen supply is replenished and oxygen demand decreases. Laurence Irving (1933) introduced the idea of "oxygen debt" to the field of diving physiology and was the founder of the concept behind having to "repay" the oxygen debt incurred during a dive after lactate builds up in the muscles. For diving specifically, he noted that using an oxygen debt method would only be effective for a few individual dives, but could not be used to explain repetitive dives performed by marine mammals.

The aerobic dive limit (ADL) represents the amount of time that a marine mammal can dive aerobically before switching to anaerobic metabolism and is directly measured by obtaining post-dive lactate levels (Kooyman et al., 1980). However, direct measurements of lactate levels from freely-diving animals have historically been challenging to obtain. Kooyman et al. (1980) was able to rectify the challenge by taking lactate measurements from freely-diving Weddell seals at ice holes that were isolated beyond the ability of the seals to reach another ice hole. Thus, the researchers knew exactly where the animals would surface to respire and were able to collect samples.

Besides the Weddell seal (*Leptonychotes weddellii*) (Kooyman et al., 1980) the only other freely-diving animal to have these direct measurements are emperor penguins

(*Aptenodytes forsteri*) (Ponganis et al., 1997b; Williams et al., 2011). Direct measurements in these studies were performed with an experimental design similar to that of Kooyman et al. (1980). Ponganis et al. (1997b) determined the ADL for freely diving, wild emperor penguins to be 5.6 minutes.

Other studies have obtained direct measurements of post-dive blood lactate concentrations, however, the animals measured were not wild, but trained to voluntarily dive (Ponganis et al. 1997a; Ponganis et al. 1997c; Shaffer et al. 1997; Williams et al. 1999). Lactate levels in trained California sea lions (*Zalophus californianus*) were directly measured and ADL was determined to be 2.3 minutes (Ponganis et al., 1997a). Post-dive lactate levels have also been directly measured in trained bottlenose dolphins (*Tursiops truncatus*) and researchers determined the ADL to be 4.5 minutes when sedentary, and decreasing to 1.3 minutes when swimming at 2.9 m s^{-1} continuously (Williams et al., 1999). Other researchers reported directly measured lactate levels in trained belugas (*Delphinapterus leucas*) and reported an ADL of 9-10 minutes (Shaffer et al., 1997). Lastly, trained Baikal seals (*Phoca sibirica*) were measured directly while in diving tanks and were reported to have a 16-minute ADL (Ponganis et al., 1997c). In other species, it is not always logistically feasible to obtain ADL measurements and so only a handful of animals have ever been directly measured for lactate levels.

Due to the difficulty obtaining direct lactate and metabolic measurements and duplicating the experimental set up in Kooyman et al. (1980), many researchers tried calculating the ADL as a proxy for directly measuring the ADL (Castellini, 2012). The calculated aerobic dive limit (cADL) is estimated from measurements of the O_2 stores

and the O_2 consumption rate (Butler and Jones, 1997). But, Butler and Jones (1997) argued that there is a true difference in cADL and directly measured lactate levels for ADL. In fact, Ponganis et al. (2010) noted that the cADL underestimated the ADL in emperor penguins by 35 to 55% (Table 1). Williams et al. (1999) also found cADL underestimations of 23-36% from the measured ADL in bottlenose dolphins, and pointed out that it is difficult to estimate metabolic rates to calculate ADL without knowing the behavior and/or the activity level of the animal. However, Shaffer et al. (1997) and Ponganis et al. (1997c) reported similar ADL and cADL in beluga whales and Baikal seals, respectively, but did not measure metabolic rate (Table 1). The differences discussed here between the actual ADL and cADL demonstrate not only our lack in data, but the logistical difficulty associated with collecting this type of data. Despite all of this, the cADL is still considered an important and common tool used when trying to understand the diving behavior, physiology, and ecology in marine mammals.

Table 1. Summary of direct lactate measurements in the few marine mammals directly measured. The underestimations in cADL compared to actual ADL measurements illustrate the need for direct measurement in the field of diving physiology and other genres utilizing field estimates and predictive models.

Marine Mammal	Researchers	ADL (min.)	cADL
California sea lion (*Zalopholus californianus*)	Ponganis et al. 1997a	2.3	35-55% less
Bottlenose dolphin (*Tursiops truncates*)	Williams et al. 1999	4.5	23-36% less
Beluga whale (*Delphinapterus leucas*)	Shaffer et al. 1997	9-10	Same
Baikal seal (*Phoca sibirica*)	Ponganis et al. 1997c	16	Same

The dive response

A seminal concept in diving physiology is that all air-breathing vertebrates exhibit a "dive response" which includes cessation of breathing (apnea), a reduction of heart rate upon submergence (bradycardia), and reduced blood flow to peripheral tissues (peripheral vasoconstriction) (Scholander, 1940). The dive and exercise responses are contradicting each other when it comes to changes in heart rate, vasodilation to the working muscle groups, and the effect of ventilation and oxygen consumption as work rate increases (Burton et al., 2004). Even at rest, energy is consumed in order to maintain organ function. As all air-breathing vertebrates experience a dive response, several attempts have been made to explain the physiological differences between marine and terrestrial mammals. However, the question remains: how can marine mammals manage the contradicting physiological responses while diving and exercising?

Marine mammals managing metabolic costs

Marine mammals employ various strategies to minimize metabolic costs while taking advantage of their aquatic niche. Animals inhabiting an aquatic environment encounter constraints that terrestrial mammals do not experience, such as light availability, pressure changes, and temperature variations. Therefore, in order to perform dives necessary to find food and avoid predators, marine mammals have evolved physiological traits uniquely suited to meet energetic demands in an aquatic environment (Wienecke et al., 2007).

Marine mammals have larger blood volumes, higher concentrations of hemoglobin in the red blood cells, and higher concentrations of myoglobin in the muscles in which they store oxygen as compared with their terrestrial counterparts (Lenfant et al., 1969; Kooyman and Ponganis, 1997; Kooyman, 2009). Davis et al. (2004) speculated that high myoglobin concentrations in the muscles of air-breathing, diving vertebrates, as well as other traits mentioned above to maintain aerobic metabolism at low partial pressures of oxygen, have allowed them to sustain prolonged periods underwater while exercising.

Additionally, some marine mammals tend to have slightly larger lung volumes compared to that of terrestrial mammals (Kooyman, 1973). In fact, the sea otter (*Enhydra lutris*) has one of the largest O_2 stores of all marine mammals because of its relative lung volume compared to other marine mammals. However, while the human lung is an important oxygen store, this is not the case for all marine mammals (Kooyman, 2009). During the evolution of vertebrates, the main function of the lung was to exchange gases

between the blood and the air, but this function is diminished when marine mammals dive to depth (Kooyman, 2009) because the animal is breath-holding. Indeed, the alveoli compress and collapse completely when diving, shunting the blood from the lungs and ceasing gas exchange. So what other physiological traits are employed by marine mammals to function with only a finite oxygen resource in one breath of air?

Davis and Kanatous (1999) questioned how oxygen is distributed to the tissues in a way that would maximize the ADL even when the animal was exercising. When modeling oxygen transport in Weddell seals, Davis and Kanatous (1999) believed that the oxygen stores in myoglobin alone were not enough to support oxygen consumption in the muscle tissues. Based on their modeling results, they were able to show that subtle adjustments to cardiac output and peripheral vasoconstriction could be made at the beginning of a dive to match the animal's level of activity and maximize the ADL (Davis and Kanatous, 1999).

Furthermore, blood flow is reduced to the visceral organs, but homeostasis is maintained (Davis et al., 1983). The brain, however, must receive an adequate supply of oxygen at all times, and blood flow to the brain is maintained. Irving (1933) speculated that some marine mammals such as seals must have an increased buffering capacity in their muscles (Irving, 1933) and proposed that seals were much less sensitive to carbon dioxide build up as compared with humans (Irving et al., 1935). Later, Elsner (1970) provided evidence that Weddell seals, and seals in general, may have adapted the ability to tolerate low oxygen in the brain and had a lower oxygen consumption rate when diving. These known physiological responses allow Weddell seals to conserve energy and

maintain homeostasis while diving. Still there are gaps in the knowledge whereby these responses are directly measured and this study aims to fill in some of those gaps.

In addition to the dive response, there are additional physiological traits used by marine mammals in regards to ADL to extend aerobic dive time. Irving (1933) wanted to better understand how marine mammals could dive for long periods of time and theorized that they were able to do so by employing a low metabolic rate. Castellini (1981) found that some enzymes in marine mammals were adapted to better process lactic acid in the heart, liver, and muscle when the animal was recovering from a dive on the surface. Castellini et al. (1985) studied metabolism in gray seals (*Halichoerus grypus*) and concluded that under low oxygen conditions, the organs and muscles decreased metabolic rate. The seals did not incur any more oxygen debt when exercising and diving than when resting on the surface. Another study found that the metabolic rate of diving decreased as dive duration increased and demonstrated that most dives were well within the ADL and that the cost of diving was incredibly low (Castellini et al., 1992).

What are some of the methods marine mammals use to lower their metabolic rates thereby saving energy costs and extending the ADL? In addition to increased concentrations of blood and muscle constituents, there are some known strategies such as blubber usage in buoyancy at the surface (Kooyman, 1973) and for thermoregulation (Williams et al., 1999), and burst-and-glide methods in swimming (Davis et al., 2001). However, these only explain a fraction of the extended dive durations performed by marine mammals in their extreme environments.

For example, one of the most apparent features marine mammals have developed is a thick layer of blubber that encases the body. Blubber constitutes 20-30% of total body weight of a marine mammal, resulting in additional buoyancy (Kooyman, 1973). By not having to control buoyancy at the surface, some marine mammals derive additional savings in metabolic costs (Webb et al., 1998). This feature also allows marine mammals to conserve metabolic energy normally required to replace the body heat lost to the water via thermoregulation.

Additionally, the vascular system also plays an important role in thermoregulation in some marine mammals (Williams et al., 1999). In the bottle-nosed dolphin, countercurrent arrangement of blood vessels in the peripheral fins (pectoral, dorsal and caudal) allows for diffusion of heat from the warm blood coming from the core to the blood returning from the periphery (Williams et al., 1999). Heat accumulates in the core of marine mammals, especially during exercise, and must be dispersed to maintain homeostasis. Peripheral blood flow is decreased during diving to conserve oxygen, but needs to be increased to allow for heat dissipation during thermoregulation. It would seem that the cardiovascular system in some marine mammals have a conflict between the dive response and thermoregulation. However, Williams et al. (2000) discovered that this conflict is attenuated in bottlenose dolphins. They concluded that rather than conflict with the dive response, heat dissipation is delayed until the animal surfaces and the need for oxygen conservation is reduced (Williams et al., 1999). Being able to thermoregulate more efficiently via countercurrent heat exchange reduces the metabolic costs of heating and cooling the animal in an environment with high thermal conductivity.

Another energetic consideration that must be mitigated in marine mammals is the metabolic cost of swimming. When diving to depth, many marine mammals can use prolonged gliding techniques when negatively or neutrally buoyant to conserve energy and can use swimming strokes to ascend to the surface until positively buoyant once again (Davis et al., 2001). When air spaces are compressed, body volume is decreased without a change in body mass, which results in the body potentially becoming negatively buoyant depending on the density of the animal. Therefore, diving animals can conserve energy by gliding on descent instead of actively stroking (Williams et al., 2000). In fact, Weddell seals demonstrated a 9.2 to 59.6 % reduction in estimated energetic costs when modifying locomotor patterns, increasing their aerobic dive duration (Williams et al., 2000).

Forced submergence vs. freely-diving studies

Historically, diving physiology studies were usually performed with animals in a laboratory setting (Bert, 1870; Richet, 1899; Irving, 1934; Irving et al., 1935). Variables such as dive time and depth were easier to manipulate in the lab, and precise measurements of heart rate and blood flow could be more easily observed (Irving et al., 1935; Scholander, 1940; Irving et al., 1941). These early studies often involved forcibly submerging the animals and measuring their responses. Forced submergence is a method where the animals are physically restrained during the “dive”. It was suggested that forced submersion includes a stress component and that the response may be maximal (Irving, 1934). Studies on voluntary- diving wild animals began in the 1960’s which

allowed physiological responses to be measured during natural dives (Castellini, 2012). However, some researchers continued to study animals using forced diving techniques to obtain physiological data that were logistically difficult in the field. Others used captive animals that were trained to participate voluntarily, which allowed controlled studies of stress free physiological responses (Ponganis et al. 1997a; Ponganis et al. 1997c; Shaffer et al. 1997; Williams et al. 1999). These studies have indicated that the magnitude of the response differs considerably in forced versus voluntary dives (Scholander, 1940; Kooyman and Campbell, 1972; Kooyman et al., 1980; Jobsis et al., 2001; McCulloch et al., 2010; Panneton et al., 2010). Results from forced dive studies may be misleading because the data collected may not be the natural response of a freely diving animal in the wild (Kooyman and Campbell, 1972). Therefore, it is imperative to understand how these animals manage physiological responses in their natural environment.

In order to improve our understanding of how marine mammals adjust muscle blood flow during diving to increase dive time, we developed and tested a tag with oxygen sensors that attempted to measure O_2 saturation in the muscle of freely deep-diving elephant seals. Northern elephant seals are some of the deepest diving marine mammals, and current understanding of their diving physiology is rudimentary at best while their diving patterns and life history have been well studied (Le Boeuf et al., 1986; Le Boeuf et al., 1988; Andrews et al., 1997). This species was ideal as they return to Año Nuevo every spring to molt on the beach. If trans-located to a remote place in the ocean before they have molted they return to the same beach and perform long and deep dives during the transit back to the beach. These characteristics made the elephant seal an ideal

candidate for this study as we have the opportunity to study this freely diving seal in its natural, sometimes extreme, environment. The sensors were incorporated into the data-logging device and tested in the elephant seal in collaboration with Dr. Daniel Costa (University of California-Santa Cruz). Understanding how foraging marine mammals manage metabolic costs with finite stores of oxygen is paramount to understanding their success in an extreme environment.

Objectives

The purpose of this study aimed at using an archival tag and implanted sensor system to measure physiological variables in freely deep-diving northern elephant seals. The study will improve our understanding how physiological mechanisms function during breath-hold diving in marine mammals. The study also aimed to increase our understanding of basic animal physiology and physiological adaptations to the marine environment and provides information on how marine mammals optimize their diving response.

Following the sensor and data logger development, we set out to (1) test a long-held assumption that oximeter use in marine mammals could be calibrated with terrestrial mammal blood. We tested that assumption by comparing the absorption spectra of human hemoglobin (Hb) to that of a few species of marine mammals. The Northern elephant seal was included in the comparative study so that we could properly calibrate our oximeter for field use. The (2) surgical techniques for implanting the sensors were developed. We believed that a minimally invasive procedure would be extremely useful in future studies

as it would help develop systems that can measure a variety of physiological parameters in other species. Once the surgical methods had been developed, we (3) implanted the sensor and deployed the data logger in the Northern elephant seal.

The objectives and subsequent chapters of this thesis are as follows:

1. Test an assumption: oximeters can be calibrated using terrestrial mammal blood for use in marine mammals.
2. Develop surgical methods and calibrate sensors and data loggers.
3. Implant and deploy the sensor and data logger in the Northern elephant seal.

CHAPTER 1 – HEMOGLOBIN ABSORPTION SPECTRA IN MARINE MAMMALS: A COMPARATIVE STUDY

1. INTRODUCTION

In all mammals, O_2 is carried through the cardiovascular system by the protein hemoglobin (Hb). Each Hb carries up to 4 O_2 molecules to the tissues where the O_2 is released to support aerobic metabolism. The Hb undergoes conformational changes as each O_2 molecule binds, which affects its O_2 affinity and optical properties. When all binding sites are occupied and the Hb fully saturated (HbO_2) it is bright red, whereas deoxygenated Hb (HbR) is a darker red.

The difference in optical properties between HbO_2 and HbR is determined by how much light is absorbed, transmitted, and scattered by the protein as it changes shape. Thus, the absorbance spectra for HbO_2 and HbR are distinct and have been of clinical importance to determine arterial O_2 saturation (Horecker, 1943; Kuenstner and Norris, 1994; Rolfe, 2000). Where absorption for HbO_2 and HbR is equal in the near-infrared region is called the isobestic point and is ~800 nm in humans (Mancini et al., 1994; Rolfe, 2000; Williams et al., 2011). As the total absorbance of any wavelength depends on the Hb concentration ([Hb]), the isobestic point is useful to assess changes in hemoglobin concentration. This is of particular importance as Hb concentration varies among and within mammal species (Perutz, 1983).

The absorption spectra for human Hb and other terrestrial mammals such as the rat, dog, and cow have been well characterized (Zijlstra and Buursma, 1987; Mancini et al., 1994; Zijlstra et al., 1994; Rolfe, 2000). Some slight differences in absorption spectra

of Hb have been found among terrestrial mammals (Zijlstra and Buursma, 1987; Zijlstra et al., 1994). While the absorption spectra for human and dog were not different (Zijlstra and Buursma, 1987), differences were found when comparing human and rat blood that were large enough to cause significant differences in spectrophotometric analysis of blood (Zijlstra et al., 1994). Therefore, Zijlstra et al. (1994) concluded that spectrophotometric measurements for rats should be made on data collected from absorption spectra in the same species.

Marine mammals have several traits that enable them to stay underwater while foraging for food (Kooyman, 1989; Davis et al., 2004). There have been hematological studies in marine mammals to determine the cardiovascular adaptations that help increase time spent underwater as compared to their terrestrial counterparts, including investigations to measure hematocrit levels, red blood cell counts, and 2, 3-diphosphoglycerate (DPG) concentration (Lenfant et al., 1969; Kooyman et al., 1980; Snyder, 1983; Castellini et al., 1986; Hedrick and Duffield, 1991; Castellini et al., 1996; Castellini, 1999; Meiselman et al., 1999; Castellini and Castellini, 2002; Castellini, 2012). Some species of mammals have structurally different Hb polymorphisms as demonstrated by electrophoresis (Lenfant et al., 1969; Wells and Brennan, 1979; Pérez-Suárez et al., 1986). These isoforms differ in their O_2 affinity and Bohr shift dynamics (Wells and Brennan, 1979). For example, "fast" Hb have greater O_2 affinity, while the Bohr shift is more pronounced in "slow" Hb (Wells and Brennan, 1979). As marine mammals appear to have structurally modified Hb to assist prolonged apnea (Wells and Brennan, 1979) this may also alter the optical properties of this protein, thereby affecting

the results of spectrophotometric evaluations. However, no study has investigated the spectrophotometric properties of Hb in marine mammals (Farmer et al., 1973).

The absorption spectra of HbO_2 and HbR are used to estimate arterial O_2 saturation in conventional oximetry, where the change in absorption is used to determine O_2 saturation. In addition, the isobestic point is used to account for changes in Hb concentration (Mancini et al., 1994; Guyton et al., 1995; Williams et al., 2011). Most oximeters used in research or clinical medicine have been calibrated against terrestrial species such as human volunteers, dogs, horses and birds (Matthews et al., 2003; Fahlman, 2008). Thus, the use of an oximeter across different species relies on the assumption that the Hb absorption spectra and isobestic point are similar across mammals. Consequently, the assumption that the absorbance spectra are identical for all mammals should be tested before spectroscopy to study O_2 saturation in marine mammals is used.

The objective of this study was to determine if the absorption spectra for HbR and HbO_2 are similar across marine mammal species and the same as those in humans. We hypothesized that Hb absorption spectra and the isobestic point of 800 nm is the same in marine mammals as it is in humans. To test the hypothesis, we isolated Hb from a variety of species and determined the HbO_2 and HbR spectra. We validated our methods by comparing the absorbance spectra from human Hb with those previously published (Horecker, 1943; Kuenstner and Norris, 1994; Rolfe, 2000). Our results suggest that the HbO_2 and HbR from killer whales (*Orcinus orca*), short-finned pilot whales

(*Globicephala macrorhynchus*), beluga whales (*Delphinapterus leucas*), and Northern elephant seal (*Mirounga angustirostris*) are similar and all have the same isobestic point.

2. MATERIALS AND METHODS

All human (IRB approval # 123-11) or animal procedures (IACUC AUP #03-11) were approved by the Texas A&M University – Corpus Christi ethics and animal care committee.

2.1 Blood sampling

For human blood, 30 ml whole blood samples were obtained in 10 ml lithium heparin vacutainers (REF# 367880 BD Vacutainer, Franklin Lakes, NJ, USA) from consenting volunteers. The human blood samples were stored at 4°C overnight to maintain consistency when comparing against samples from marine mammal samples that were shipped on ice overnight.

Approximately 30 ml (venous, arterial, or mixed venous/arterial blood) was collected opportunistically from 5 individual killer whales, short-finned pilot whales, and beluga whales during scheduled blood draws from three Sea World facilities. Blood samples were obtained from 2 free ranging Northern elephant seals from Año Nuevo, CA (NMFS permit #1463). All blood samples were placed on ice and couriered priority overnight in a shipping cooler to the Comparative Physiology Laboratory at Texas A&M University - Corpus Christi.

2.2 Hemoglobin isolation

Hemoglobin from whole blood was isolated through a process of centrifugation and dialysis (protocol provided by and modified from Akito Nakagawa, Massachusetts General Hospital) to determine the absorbance spectra of Hb. The blood sample was transferred into a 50 ml centrifugation tube. The sample was centrifuged for 15 minutes at 2000 g (Beckman model JA-21 centrifuge, ≈5000 rpm, rotor radius = 10.2 cm). After centrifugation, the plasma and buffy layer and supernatant were removed and discarded. Next, a volume of phosphate buffered saline (PBS at pH = 7.4) equal to the discarded supernatant was added and the sample centrifuged again. This procedure continued until the supernatant was clear, usually 3-4 times. After the supernatant had been removed following the last centrifugation, cold (4°C) distilled water was added to lyse the red blood cells and release the Hb. The Hb solution was centrifuged for one hour at 18,500 g (≈15,000 rpm). The supernatant, containing the Hb and distilled water, was transferred to a new centrifuge tube and again centrifuged for an hour at 18,500 g. The centrifugation procedure was repeated a total of 3-4 times until it determined visually that all cellular debris was removed.

After the final round of centrifugation, the sample was transferred into dialysis tubing (Spectra/Por Dialysis Membrane MWCO: 6-8,000, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). To remove any byproducts from the manufacturing process and make it more flexible, the tubing was soaked in DI water for 30 minutes prior to use. The Hb solution was transferred to the dialysis tubing, both ends were clamped, and the tubing placed into 1000 ml of PBS where it remained refrigerated at 4°C

overnight. The PBS solution was exchanged after 2, 4, 16 and 18 hrs. The dialyzed sample was filtered through a 0.45 µL and a 0.2 µL syringe filter.

2.3 Tonometer and Spectrophotometer

A custom-built tonometer was used to oxygenate and deoxygenate the isolated Hb sample (30 ml). The tonometer consisted of a pair of centrifuge tubes (50 ml) that held the Hb sample in a water bath at 37° C to maintain a constant temperature. Each tube was agitated while in the water bath at 350 rpm to distribute the Hb solution into a thin film while the tube was flooded by gas to either oxygenate or deoxygenate the sample. The 30 ml Hb sample was divided into two equal volumes of 15 ml each and these were simultaneously oxy- and deoxygenated. Thus, each paired sample assured that the Hb concentration was the same between paired samples. The Hb samples were deoxygenated and oxygenated by flushing the tonometer with a 95% N_2/5% CO_2 or a 95% O_2/5% CO_2 gas mixture, respectively. To prevent dehydrating the Hb sample, the dry gas was bubbled through distilled water before reaching the tonometer. After each sample was prepared by tonometry for 30 minutes, the samples were then transferred to a 3 ml cuvette under gas-tight conditions. The absorbance spectra were measured in a UV-Vis spectrophotometer in transmittance mode at 1 nm increments from 600-1000 nm (Shimadzu UV-1800, Shimadzu Corporation, Kyoto, Japan). The spectrophotometer corrected the Hb sample spectrum using a reference sample of PBS solution. Another subsample was used to determine the PO_2 and pH (Sevenmulti model, Mettler Toledo AG, Schwerzenbach, Switzerland).

2.4 Tonometer time course

Initial trials suggested that dehydration of the Hb sample occurred despite bubbling the dry gas through distilled water. For this reason we determined the shortest duration in the tonometer to achieve adequate PO_2 values that represent fully oxygenated (PO_2 > 200 mmHg) and deoxygenated (PO_2 < 10 mmHg) Hb. Initially, 15 ml of PBS alone was prepared in the tonometer using the procedure as described for 60 minutes. Every 15 minutes, the PO_2 (Fire Sting O_2 fiber optic mini oxygen electrode, PyroScience GmbH, Aachen, Germany) and pH were measured and the PBS sample was weighed.

3. RESULTS

3.1 Tonometer time course

The mean PO_2, pH, and weights of the PBS are summarized in Table 1 and 2 for both oxygenated and deoxygenated PBS (Table 2) and Hb/PBS (Table 3) samples. Any water loss was assumed to be distilled water. At 37° C, the density of pure water is 0.99 g ml^{-1}, and assumed it to be 1.00 g ml^{-1} for the purposes of this study. During the first 30 minutes (0-30 minutes) the mean water loss was 1.36 ± 0.33 ml for oxygenated PBS and 1.55 ± 0.26 ml for deoxygenated PBS. Table 3 shows the same variables for a single Hb sample in PBS. The water loss for the first 30 minutes was the same at 1.57 ml and 1.72 ml for the HbO2 and HbR samples. Water loss for samples between PBS only trials and isolated human Hb was not significantly different when oxygenated ($p = 0.61$) or deoxygenated ($p = 0.59$).

Table 2. Mean PO_2, pH, and weight of PBS following each 15 min time increment of tonometry. Values are means and standard deviation of the 4 trials.

Time (min)	Oxygenated			Deoxygenated		
	PO_2 (mmHg)	**pH**	**Weight PBS (g)**	**PO_2 (mmHg)**	**pH**	**Weight PBS (g)**
0	210.8 ± 14.2	7.35 ± 0.04	14.9 ± 0.2	215.0 ± 15.0	7.31 ± 0.02	15.0 ± 0.1
15	700.5 ± 36.7	6.62 ± 0.03	14.2 ± 0.2	4.0 ± 0.9	7.34 ± 0.02	14.2 ± 0.2
30	716.2 ± 40.5	6.60 ± 0.03	13.5 ± 0.2	3.8 ± 0.9	7.36 ± 0.02	13.4 ± 03
45	723.6 ± 38.9	6.61 ± 0.03	12.8 ± 0.3	3.6 ± 1.4	7.37 ± 0.01	12.7 ± 0.4
60	734.5 ± 53.9	6.63 ± 0.04	11.9 ± 0.3	3.5 ± 0.7	7.38 ± 0.02	11.9 ± 0.4

Table 3. The summary of PO_2, pH, and weight values of isolated human Hb following each 15 min time increment of tonometry.

Time (min)	Oxygenated			Deoxygenated		
	PO_2 (mmHg)	**pH**	**Weight PBS (g)**	**PO_2 (mmHg)**	**pH**	**Weight PBS (g)**
0	221.1	7.08	14.9	231.1	7.03	14.7
15	701.5	6.64	14.2	10.97	7.14	13.8
30	717.5	6.67	13.4	5.503	7.20	13.0
45	729.3	6.71	12.4	2.994	7.21	12.6
60	724.7	6.71	11.7	2.163	7.22	12.1

The initial pH of the PBS decreased slightly from ~7.4 to ~7.3 when placed in the water bath. This decrease in pH was most likely caused by an increase in the temperature of the PBS from storage at 4°C to 37°C in the water bath (Bates, 1973). The PO_2 and pH values for the oxygenated PBS trials and for isolated Hb were similar at each 15-minute interval (Tables 2 and 3, respectively) with no difference in oxygenated PBS or isolated

human Hb (PO2 $p = 0.99$; pH $p = 0.99$). In addition, there were no differences in the deoxygenated PO_2 between the PBS and isolated Hb ($p = 0.94$), but there was a significant difference in pH values between the two ($p < 0.001$).

After 15 minutes in the tonometer, the PO_2 of the oxygenated PBS and HbO_2 were > 700 mmHg, a value sufficiently high to assume saturation. For the deoxygenated sample, a PO_2 < 10 mmHg required at least 30 minutes in the tonometer. In addition, many Hb samples began to coagulate after being in the tonometer for longer than 30 minutes, and in some cases aggregations could be found after only 15 minutes. For these reasons, we opted to run all samples for 30 minutes to allow proper oxygenation and deoxygenation of the samples and to prevent significant dehydration of the samples.

3.2 Isolated human Hb spectra

The human absorption spectra (Figure 1) closely resembled those previously published using a range of different methods for isolating Hb (Horecker, 1943; Kuenstner and Norris, 1994; Rolfe, 2000).

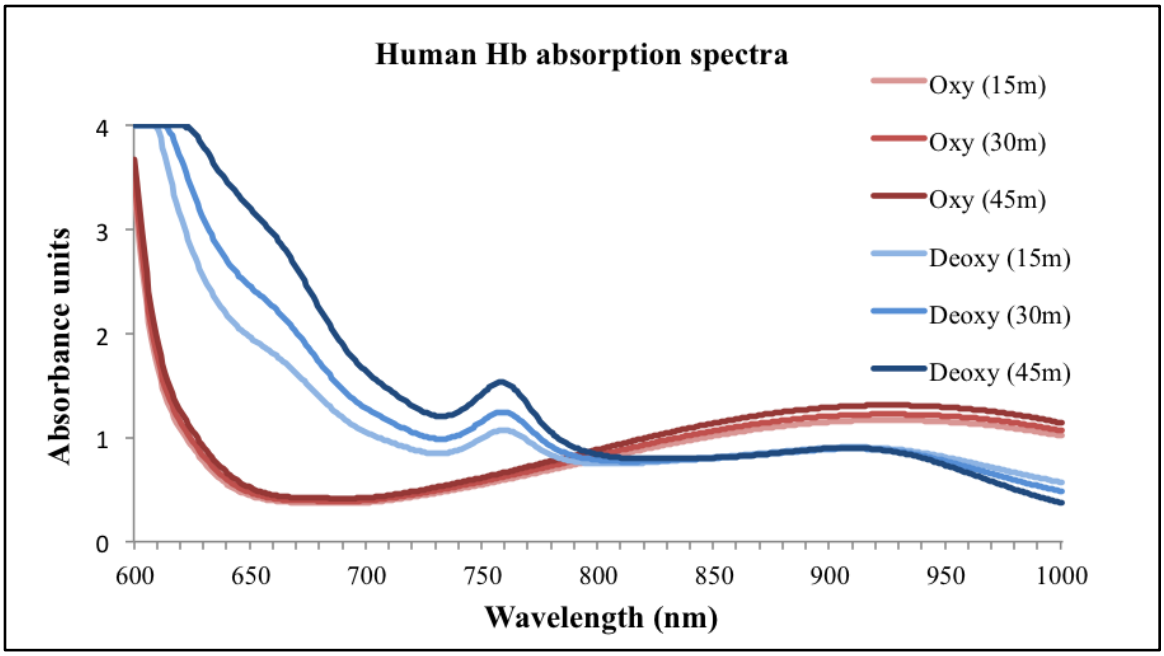

Figure 1. Hb absorption spectra measured in 15 minute increments. The spacing between the spectra indicates further deoxygenation or oxygenation. The isosbestic point is consistently ~800 nm as in published works for human Hb.

3.3 Marine mammal Hb

Table 4 shows the mean PO_2 and pH for each HbR and HbO_2 samples from each marine mammal species investigated. Deoxygenating beluga blood samples was problematic as we were unable to deoxygenate any of the beluga samples to <10 mmHg; therefore, we only used samples with a PO_2 < 22 mmHg were used for further analysis. The absorption spectra from one representative animal sample (1 animal sample out of the 5 samples analyzed from each species) are shown in Figure 2 with the PO_2 and pH for those animals in parenthesis in Table 4. The spectra are similar and the isobestic points were ~800 nm for all species.

Table 4. The mean and standard deviation of PO_2 and pH values for all marine mammal samples included in study after 30 minutes of tonometry. In the parentheses are the PO_2 and pH values for the individual representative marine mammal samples included in study corresponding to Figure 2.

Species	**n**	**Oxy pH**	**Deoxy pH**	**Oxy PO_2 (mmHg)**	**Deoxy PO_2 (mmHg)**
Orca	5	6.69 ± 0.12 (6.78)	6.74 ± 0.13 (6.87)	496.9 ± 27.8 (500.0)	8.3 ± 6.8 (3.8)
Pilot whale	5	6.78 ± 0.02 (6.78)	6.81 ± 0.03 (6.84)	314.2 ± 132.9 (448.7)	5.2 ± 3.1 (3.8)
Beluga	2	6.84 ± 0.00 (6.85)	6.87 ± 0.02 (6.90)	703.5 ± 38.5 (730.7)	21.5 ± 0.02 (21.5)
N. elephant seal	2	6.85 ± 0.00 (6.85)	6.89 ± 0.01 (6.89)	448.6 ± 107.8 (524.8)	14.2 ± 2.6 (12.3)

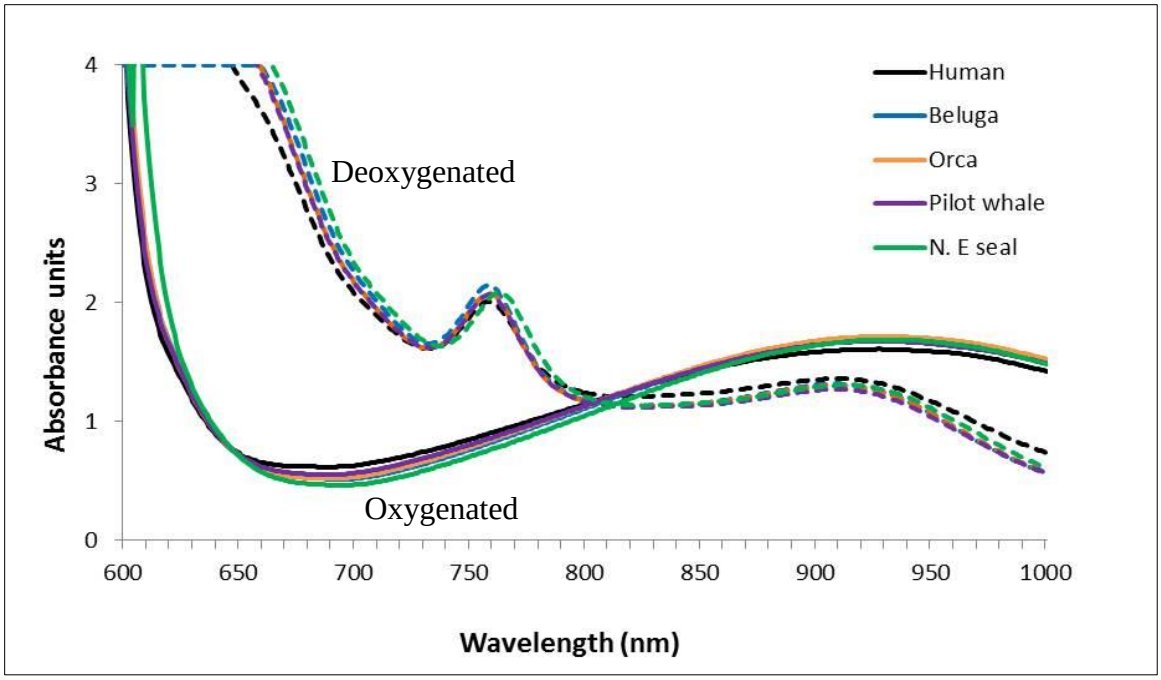

Figure 2. The comparison of human Hb and marine mammal Hb absorption spectra. The dotted lines are HbR spectra, and solid lines areHbO_2 spectra. Consistently, the absorption spectra are very similar to human Hb with an isosbestic point of ~800 nm for all species in the study.

4. DISCUSSION

We compared the absorbance spectra of human and marine mammals Hb. The HbR, HbO_2, and isobestic points were identical between species. Human absorbance spectra after subjecting hemoglobin to 30 minutes of manipulation with the tonometer was identical to those previously published. In addition, most of the marine mammal Hb samples were properly oxygenated or deoxygenated using our methods, with the exception of the belugas, allowing us to compare the absorbance spectra within and between animals and species.

4.1 PBS Trials: Effects of drying out samples

In the current study, we found a temporal effect on the dehydration of our samples during tonometry. Increased tonometry duration increased the amount of sample lost to dehydration. Despite bubbling the dry gas through distilled water to humidify it, the Hb sample still lost water while being tonometered. Dehydration of the Hb sample would significantly affect the [Hb] and thereby the absorbance. We were not able to measure the [Hb] using Drabkin's reagent, but as the HbO_2 and HbR were compared from the same initial blood sample, and as either oxygenating or deoxygenating the sample did not affect the rate of water loss, the direct comparison should be valid.

Hemoglobin concentrations ([Hb]) have been observed to vary both within and between species (Kooyman, 1989; Hedrick and Duffield, 1991), and also due to differences in the isolation techniques. The [Hb] will alter the absorbance spectra by shifting it up or down as seen in the current study. While attempts were made to measure [Hb] these results were unreliable. Despite our inability to measure the [Hb] between samples, each HbR and HbO_2 sample was a subsample of the isolated Hb and therefore had the same concentration before being tonometered. As we showed that the magnitude of dehydration was only affected by the duration in the tonometer, each HbO_2 and HbR paired sample was assumed to have the same concentration.

4.2 Isolated human Hb spectra

The absorbance spectra for human Hb is well defined and was used to assess the quality of our methods (Fig. 1. Horecker, 1943; Kuenstner and Norris, 1994; Rolfe, 2000). Our human HbO_2 and HbR spectra were almost identical to previously published data, but there were slight differences. For example, at 600-700 nm the absorbance spectras are not identical. These results are similar to previous studies where there slight differences in the absorption spectra (Horecker, 1943; Kuenstner and Norris, 1994). These differences may be the result of differing methods to isolate the Hb, where some have used detergents to lyse the cells (Horecker, 1943), while others did not (Kuenstner and Norris, 1994). However, the results from our study, using our modified isolation protocol, agreed well with data from previous work.

We did not use chemicals to deoxygenate the Hb, which may be another reason for the slight differences in absorbance spectra. In the previous published studies, tonometry with N_2 gas was used followed by the addition sodium dithionite to completely reduce the samples (Horecker, 1943; Kuenstner and Norris, 1994). Instead, our goal was to achieve a $PO_2 < 10$ mmHg for deoxygenated samples and a $PO_2 > 200$ mmHg for oxygenated samples. Our result suggests that oxygenating and deoxygenating the samples without chemical intervention provided adequate PO_2 levels to allow the human samples to be considered fully oxygenated and deoxygenated. Our methodology produced spectra that were nearly identical to the previous published methods (Fig. 1. Horecker, 1943; Kuenstner and Norris, 1994; Rolfe, 2000).

4.3 Marine mammal Hb

The average PO_2 values for all HbO_2 were > 200 mmHg. The average PO_2 for the HbR was < 10 mmHg for the killer whale and pilot whale samples. The average PO_2 for the HbR from the belugas was 60.5 ± 44.1 mmHg, and only 2 samples had a PO_2 of 21.5 mmHg. The P_{50} for beluga Hb is ~24.4 mmHg (Dhindsa et al., 1974). Because we were unable to deoxygenate 3 of the 5 beluga samples as fully, we did not include them in our analysis. However, we did keep the 2 beluga samples that were the lowest at 21.5 mmHg in the analysis. For the elephant seals, the average PO_2 for HbR was 14.2 mmHg (Table 4 and Figure 2). Although we used a PO_2 < 10 mmHg as our value for HbR, the absorption spectra for the 2 beluga whale and elephant seal HbR were similar to the other species (Figure 2). We had problems with the PO_2 electrode at the end of the study and suspect that these elevated PO_2 values may be erroneously high. This is supported by the HbR curves, which look identical to those for the other species when the PO_2 electrode was working properly. In addition, the isobestic points were still ~ 800 nm for the beluga and elephant seal samples which further supports that the high PO_2 values for the deoxygenated samples were erroneous.

Mammals express a variety of Hb molecules that differ in O_2 affinity and its relative distribution among species (Dhindsa et al., 1974; Pérez-Suárez et al., 1986). As marine mammals appear to have modified Hb to assist prolonged apnea (Wells and Brennan, 1979), we believed this could alter the optical properties of this protein, thereby affecting the results of spectrophotometric evaluations.

5. CONCLUSION

The results from our study indicate the Hb absorption spectra among humans and the marine mammal species studied are consistently similar. The isobestic point for each species is also the same as compared with humans and ~ 800 nm. Calibrating oximeters using human blood is a valid method for killer whales, beluga, short-finned pilot whales and Northern elephant seals.

CHAPTER 2: MEASUREMENT OF O_2 SATURATION IN THE NORTHERN ELEPHANT SEAL

1. INTRODUCTION

Northern elephant seals (*Mirounga angustirostris)* are one of the deepest diving marine mammals, and our current understanding of their diving physiology is rudimentary at best. Following dives to depths of 400-800 meters for 10-30 minutes, the average surface interval is ~ 2 min (Le Boeuf et al., 1988; Le Boeuf et al., 2000). They are capable of diving to depths of at least 1750 meters deep and for over 120 minutes (Hindell et al., 1992; Robinson et al., 2012).

Elephant seals spend 8-10 months at sea, but return to the rookery annually to breed and molt, and haul out of the water and onto the beach to do so (Le Boeuf et al., 1989; Le Boeuf et al., 2000). If they are captured and taken to an offshore location to be released (translocated) before they have finished the molt, they perform long and deep dives during the transit back to the same beach to finish the molt. While swimming back to beach where they molt, the seals spend an average of 87-90% submerged (Le Boeuf et al., 1986; Le Boeuf et al., 1988). Elephant seals translocated from Año Nuevo beach across Monterey Bay swim off the continental shelf and dive for an average of 17 min with a maximum duration of 30 min and to a mean depth of 305 m (Andrews et al., 1997). Thus, seasonal translocation experiments allow researchers to attach and recover equipment to study several aspects of elephant seal ecophysiology.

Theoretically, oxygen stores are insufficient to supply the elephant seal during long, repetitive and deep dives (Kooyman, 1989). According to Le Boeuf et al. (1986),

the body mass of the juvenile Northern elephant seal female (265 kg) is comparable to that of the adult Weddell seal (315 kg). Assuming that the O_2 stores and mass-specific metabolic rates are similar in the two species, the ADL should be approximately 20-25 min (Le Boeuf et al., 1986). While Weddell seals appear to perform dives beyond the ADL and then spend considerable time resting on the ice to clear the lactate (Kooyman et al., 1980), the Northern elephant seal dives continuously with only short surface intervals between dives (Le Boeuf et al., 1989; Hindell et al., 1992). Thus, the continuous diving in the Northern elephant seal indicates that the dives must be primarily aerobic, and based on this some have suggested the cADL might actually be closer to 60 minutes (Le Boeuf et al., 1986; Hindell et al., 1992). Direct field measurements of freely-diving northern elephant seal oxygen use (metabolic rate) have not been successful due to logistical constraints.

Davis and Williams (2012) determined that marine mammals maintain aerobic metabolism and that muscle blood flow is continuous and increases proportionately with heart rate and activity. In a muscle that is not perfused, the O_2 saturation would decrease continuously while reoxygenation events would indicate at least a nominal level of blood flow. Thus, measuring muscle O_2 saturation may be a viable method to determine muscle blood flow and muscle oxygenation.

In a previous study, a near infrared (NIR) spectrophotometer was used to estimate myoglobin and hemoglobin saturation in the muscle of freely diving Antarctic Weddell seals (Guyton et al., 1995). It was concluded that swimming muscles utilize endogenous O_2 during free diving. However, the recorded oxy-myoglobin absorbance suggested that

the rate of blood flow to the muscle appeared to change with dive duration, and there were periods during diving when the muscle re-saturated with O_2, possibly from a large influx of blood. As the diving patterns of the Weddell and elephant seals are different, it would be interesting to compare the management of blood flow to the muscle in these two species. For that reason, our aim was to study muscle O_2 saturation in juvenile Northern elephant seals during diving. Studying diving physiology in these marine mammals may elucidate mechanisms of metabolic gas management to improve our understanding of diving physiology.

First we (1) developed a surgical procedure to implant the sensor into the muscle with minimal impact to the muscle. This included a surgical method that minimized inflammation and prevented infection at the implantation site and insertion in a way that movement of the sensor in the muscle was minimized. Next, (2) the sensor and data logger were calibrated and tested in vivo. The sensor was implanted, and the animals observed for up to 12 hrs for signs of inflammation, infection, and changes in behavior (particularly swimming behavior) before being released. After recovery, (3) the animals were translocated and recaptured when back at Año Nuevo. The animal was observed for any signs of infection, inflammation, or sensor migration within the muscle and the sensor and data logger were then removed. The data were downloaded and analyzed. Based on these observations, we propose improvements that can be made to minimize or reduce potential trauma, improve implantation, and improve the sensor and data logger itself.

We hypothesized that there would be no signs of inflammation or infection due to the presence of the sensor and that there would be no migration of the sensor within the muscle from the time it was implanted to the time it was recovered from the animal. In addition, we believed that there would be no effect of the sensor on swimming behavior pre-and post-surgical implantation of the sensor and that the LEDs will change in response to blood flow as predicted.

2. MATERIALS AND METHODS

2.1 Description of the sensor and data logger

The sensor contained 3 LEDs in the visible red, near infrared (NIR), and infrared (IR) spectral regions (emitting at red:660, NIR:810, and IR:940 nm, respectively) and a photo detector. The sensor was designed to minimize movement in the muscle, which would minimize trauma to the animal's tissues and allow the animal to swim as normal. The diameter of the sensor end was approximately 5-7 mm in diameter and 40 mm long. The light source and silicon chip sensor was attached to the external data logger with a thin (2-3 mm diameter) flexible silver (Ag) cable (Figure 3a). As there is a lot of lateral movement between the blubber and muscle layers, the woven wires attached to both the internal sensor and external data logger allow flexibility and movement inside the animal without causing sheering damage to the muscle/blubber interface. The data logger housing was built to withstand pressure to 3000 m and had a relatively small footprint at 4 cm diameter 3.5 cm height (Figure 4a-c). The electronics were located inside the dome-

shaped housing and include the electronics board, a micro SD memory card slot, and indicator lights to ensure each component was working correctly (Figure 4d). The batteries (2 CR3032 lithium 3V coin cell) were located underneath the electronics board (Texas Instruments MSP430F5341). Measurements from the oximeter sensor were varying current (Amps) from each of the 3 LEDs recorded on a 16GB micro SD card. Additionally, pressure was recorded and converted to depth (meters).

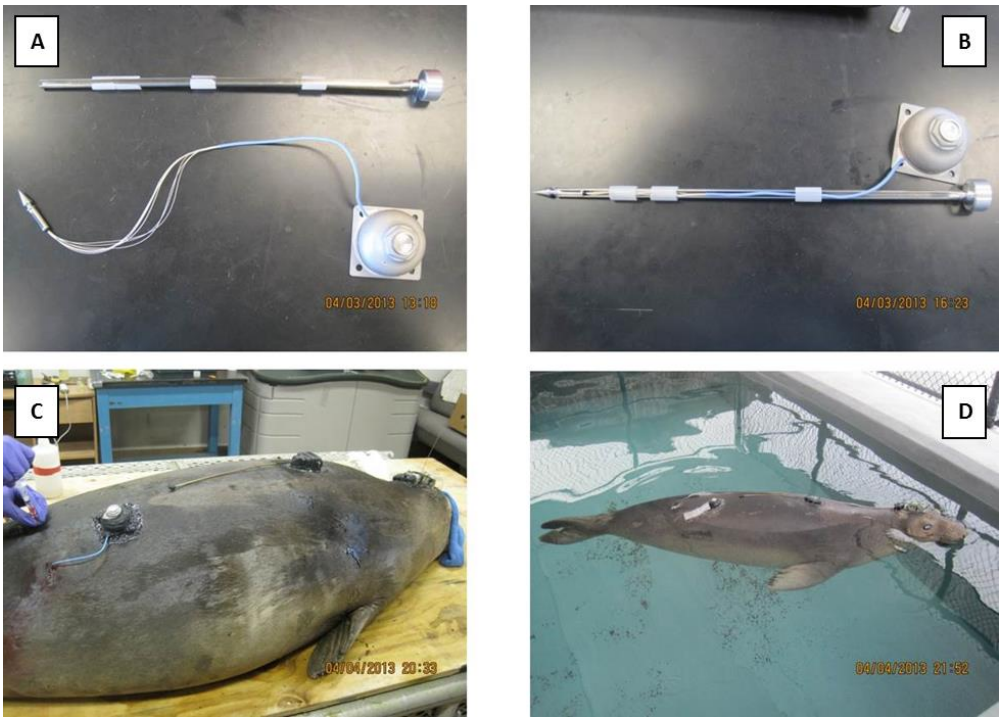

Figure 3. Pictures of the sensor and data logger implanted in the Northern elephant seal. A) The sensor attached by silver cable to the external data logger with custom stainless steel catheter used for implantation above. B) The sensor and silver cables loaded into the catheter for implantation. C) A view of the placement of the sensor after implantation and attached to the external data logger in vivo. Also shown is the satellite tag placed in a further caudel position relative to the oximeter data logger. D) One of the seals implanted with the oximeter during post-surgical recovery swimming in the recovery pool. Note the cable is covered with neoprene to avoid catching on anything in the environment.

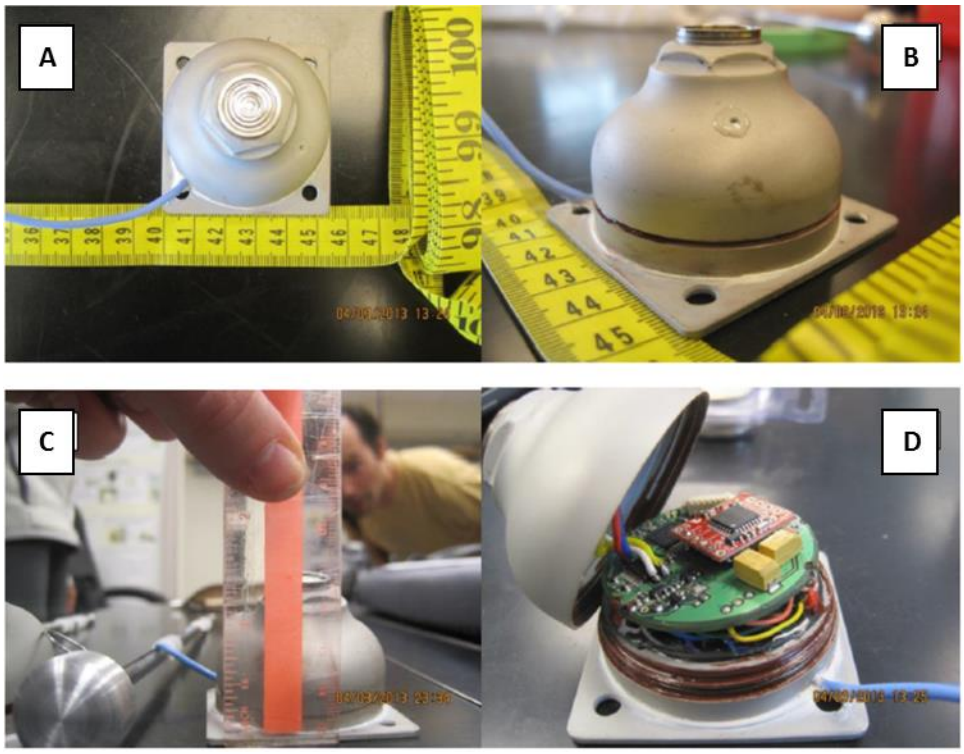

Figure 4. These pictures of the data logger are to illustrate footprint and electronics used. A and B) The square bottom of the housing allows for easy adhesion and later removal of the logger to and from the animal, while the round, dome shape is speculated to reduce drag. C). The data logger does not protrude very far from the surface of the animal which minimizes the footprint and we speculate reduces drag. D). The electronics board inside of the dome housing. The coin batteries are placed underneath the board.

2.2 Practiced implantation in carcasses

Prior to implantation into live animals, the surgical protocol needed to be developed. We did this with access to pinniped carcasses that were lost in stranding events, usually grey or harbor seals. The chance to develop and perfect the technique was given to us by Dr. Michael Moore, WHOI and Misty Neimeyer at the International Fund for Animal Welfare (Yarmouth, Massachusetts).

The carcass was laid out on a table prior to necropsy and a Terason ultrasound was used to observe obtain blubber depth. This information allowed us to determine how deeply the sensor should be placed as the aim was to implant the sensor in one of the major swimming muscles. It was important to ensure that the sensor was not placed into the blubber and that the peritoneum was not punctured. The chosen area of the carcass was shaved and cleaned, and the sensor prototype was implanted. We did this many times in different areas of dorsal muscle to determine which area would be the most suitable. Another goal was to implant the sensor quickly and effectively while minimizing any trauma to the animal that might occur during movement. Once the sensor was implanted, we would manipulate the back end of the carcass to simulate swimming movement as best we could. Then, the skin, blubber, and muscle were excised in a 5 cm^2 area and lifted away from the body so observations could be made (Figure 5).

We assessed how the sensor lay in the muscle and if there was any migration of the sensor within the muscle following the swimming muscle/back end manipulation (Figure 5). We also measured how deep the sensor needed to be implanted to be considered secured. The suture needed to secure the sensor in place was also assessed.

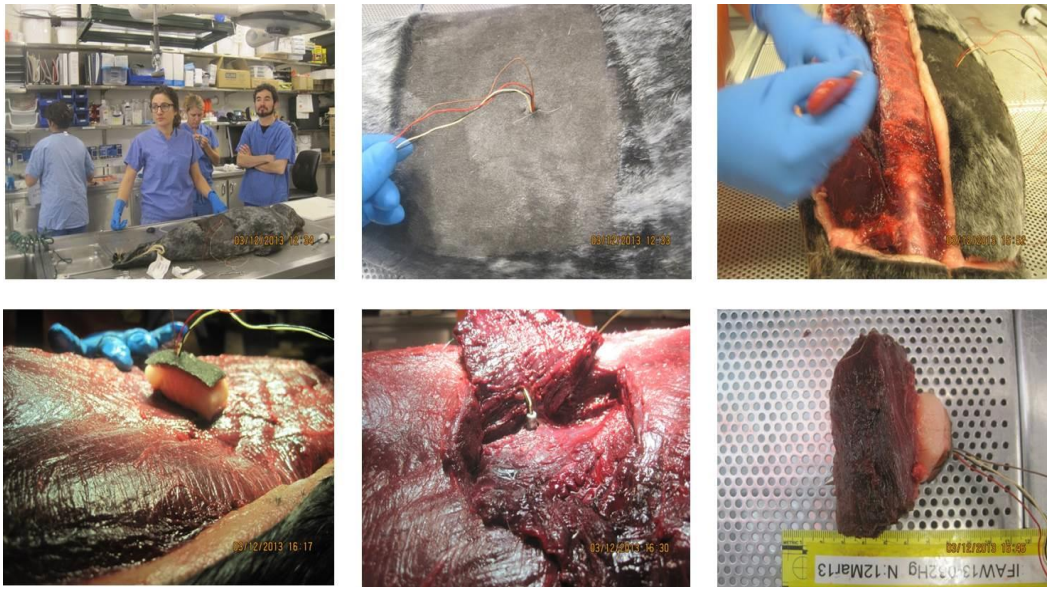

Figure 5. Pictures are from left to right. Top left is placement of the seal with surgical area cleaned and shaved. Top middle shows a prototype of the sensor implanted into the carcass of a juvenile grey seal with wires protruding. Top right is excision of the epaxial muscles along the spine on the opposite side in an attempt to identify exactly into what muscles we were implanting the sensor. Bottom left is removal of all tissue surrounding the sensor placement to better understand how the sensor would lie in the muscle and if it would migrate into or affect surrounding tissue. Bottom middle is excision of tissue at implantation site to further investigate effects of implantation. Bottom right is the skin, blubber, and muscle tissue excised around the sensor to illustrate how it was placed in the animal.

2.3 Calibration for the sensor/data logger

Human blood samples (hemoglobin) were obtained and immediately tonometered with humidified known gas concentrations of ultra-pure N_2 (for de-oxygenation) and 95% O_2/5% CO_2 (for oxygenation) for 30 minutes per sample (see Chapter 1). Hemoglobin (Hb) was used to calibrate the logger response using the assumption that the absorbance spectra are identical between Hb and myoglobin (Mb). Once the blood sample had been tonometered a 2 ml subsample was transferred using a gas tight arterial blood gas syringe

from the tonometer into a darkened blood sample tube. The tonometered blood sample vials were darkened so that no light could enter or exit. The tops of the vials were closed with rubber stoppers (BD vacutainer). Wires from the sensor were embedded in the rubber stopper and the sensor placed in the darkened vial. Blood samples of varying Hb saturation values were injected directly through the rubber stopper into the sample vial.

The samples were extracted from the tonometer, weighed, and mixed to obtain Hb saturation values of 0, 25, 50, 75, and 100% following a volumetric mixing technique (Scheid and Meyer, 1978) (Jessica Meir, personal communication). For example, a 1 ml gas-tight, glass syringe was filled with a blood sample at 0% oxygenation and another 1 ml syringe was filled with 100% oxygenated blood sample. The two syringes were mixed together for a total 2 ml blood sample at 50% oxygen saturation. Each of the final 2 ml samples were verified for P_{O2} using the Fire sting oximeter probe. Readings for each sample were taken directly from the sensor and plotted against PO_2 readings from the oxygen probe. Two attempts to calibrate the logger using whole human blood, and one attempt using isolated Hb were made. Linear regression analysis was performed (using Microsoft Excel, 2010) to confirm linearity.

2.4 Field season of implantation, deployment, and data collection

2.4.1 Animal capture

In April 2013, 5 northern elephant seals were captured from the Año Nuevo rookery under NMFS permit #1463 issued to Dr. Dan Costa. The animals were approached on foot and were chosen based on size, possession of seal id tag, and stage of

molt. The molt process had to be in the early stages so the seal would be more inclined to return to the rookery after the translocation. In addition, they were more likely to return to the rookery if they had been born and tagged there in years prior according to Dr. Luis Huckstadt, a post-doctoral fellow with UCSC (personal communication). The seals were then sedated with an injection in the posterior extradural vein with of a mixture of Tiletamine HCl and Zolazepam HCl (3ml/kg). They were kept under sedation throughout the procedure with either ketamine or telazol. The seal was weighed on the beach with a tri-pod hanging scale. Sex, approximate age, drug dosage, tag id numbers, and standard and curve lengths were recorded. Seal morphometric data are summarized in Table 5.

Table 5. Sex and morphometric data from 5 seals equipped with the oxygen sensor and data logger.

Seal ID	Sex	Weight (kg)	Standard length (cm)	Recovery time (hrs)	Return time (days)
6931	F	161	176	48	7
6939	M	198	193	48	2
G6841	M	169	186	48	4
G5944	F	173	180	8.5	6
G5882	F	162	166	4.5	6

2.4.2 Initial transport and recovery

Following the initial sedation procedure and measurements on the beach, the animal was placed into a steel transport cage designed to minimize movement. The seal

was then transported back to Long Marine Lab and placed into a caged, concrete-floored recovery area that consisted of 1m by 3 m pool filled with ambient seawater to 1.5 m deep. The pool was surrounded by a concrete deck so the animal could haul out if needed. The animals were allowed to recover for about 12 hours following capture and transport prior to surgical implantation of the probe.

2.4.3 Surgical implantation

After transport recovery overnight, the seal was once again sedated following the same protocol used during capture. The animal was moved into the laboratory and prepared for sensor implantation. Prior to the seal being sedated, the catheter, probe, and wires leading to the data logger were sterilized with Cidex OPA. The seals' hind limbs were manipulated to simulate a swimming motion to elucidate the best area for probe placement and to find the knee. Placement was determined to be dorsally between the medial and lateral line and 5-7 cm caudal to the knee.

Next, the area was wiped down to remove any dirt or feces present and scrubbed with an antimicrobial surgical scrub. A 3 cm square area of fur was removed. Ultrasound (Terason t3000/Echo with a curvilinear, model 5C2A) was used to determine blubber thickness to ensure that the probe would be placed percutaneously through the blubber and into the muscle. The shaven skin at the incision area was prepared for surgery using aseptic technique by scrubbing the area with antimicrobial soap and rinsing with sterile water and alcohol.

Surgical implantation involved a small 3-5 mm incision made in the skin with a sterile stainless steel scalpel blade to allow insertion of a 6 mm diameter peel away catheter. The incision made was smaller than the probe itself so that the probe needed to be pushed through and the skin would close behind the probe. The peel away catheter was similar to the biopsy needle approved and used by Santa Cruz scientists to obtain 0.1 gm blubber samples from elephant seals (Figure 3b). The custom built O_2 saturation sensor was implanted percutaneously by placing the catheter at a 90° angle through the blubber and into the muscle (likely *longissimus dorsi* - iliocostalis complex or the *lattissimus dorsi*). Placement is shown in Figure 3c. Once the probe/catheter had been pushed through the blubber/muscle fascia, the probe was pushed in another 2.5 to 3 cm (the length of the probe) at a 90° angle to cover the entire probe. Then the catheter was turned to a 45° angle and pushed in another 2 cm so that the probe would lie parallel within the muscle and faced caudally. The catheter was then pulled away leaving the probe in place within the muscle. A cruciate suture was used to anchor the wires leading to the data logger and to reduce the ability of the wires to slip in and out of the incision. Although the implanted sensor was designed to minimize movement in the muscle to minimize trauma to the animal's tissues, ultrasound was used again to confirm probe placement within in the muscle and to ensure that the peritoneum had not been compromised.

Once the sensor and cable were in place, the data logger was mounted externally using 5 min epoxy (Loctite) and hose-clamps and/or tie wraps glued to the fur in the mid-dorsal region. A patch of neoprene was epoxied to the fur leaving each end open and

covered the wires leading from slightly over the insertion site to the data logger. This ensured that the wires weren't pulled on and offered some protection to the incision site while allowing water to flow over and through. A satellite transmitter (4 x 3.5 cm footprint, 160 g) and radio transmitter (148-150 mHz frequency, 5 x 2 cm) were also secured to the animal's back to track the animals' location. The entire process from sedation to placement in the recovery area was 1-1.5 hours. The animals were continuously monitored while awakening from sedation.

2.4.4 Surgical recovery

Following implantation of the muscle O_2 sensor and attachment of logger and satellite and radio transmitters, the animals were held in the same fenced concrete enclosure where they were allowed to recover from initial capture and transport (Figure 3d). Any signs of inflammation, infection, or changes in swimming behavior were documented. For the first 3 seals, the data logger was started during recovery. As the battery life was much shorter than estimated and no open-ocean dive data from the oximeter logger were retrieved as the logger stopped before the animals reached the ocean. However, the ARGOS dive data were used to determine if the implantation affected natural swimming behavior. Once we had confirmed that the implant procedure did not cause major trauma, two more seals were implanted and translocated to Pt. Lobos across so that oximeter dive data could be obtained as they swam back to Año Nuevo.

2.4.5 Translocation

Following recovery, 6 to 48 hours after instrumentation, the seals were transported by truck to the opposite side of Monterey Bay, at Pt. Lobos. The seals' movements were monitored via ARGOS satellite locations, which allowed us to determine when the seal had returned to the Año Nuevo Rookery.

2.4.6 Translocation recovery and sensor and data logger retrieval

The VHF transmitters allowed us to locate the animal once it was back on the beach at Año Nuevo. Returning seals were immobilized as described previously, and another ultrasound image was obtained to determine if any movement of the probe had occurred during the seals' transit back to the rookery. Next, all instruments were carefully removed. The neoprene over the wires was cut down the middle and the implanted oximeter cable was removed by inserting the wires into a sterile custom removal device that allowed the probe to be gently pulled out of the tissue. The surrounding neoprene was left to be shed as the seal molted. The logger package was removed from the neoprene mounting system. Any signs of infection and trauma were noted.

2.4.7 Data analysis from sensor and data logger

Data from the oximeter loggers were only obtained from the last two seals, G5944 and G5882. The dive profiles of all five seals were compared using Matlab (R2011a) and Excel (Microsoft, 2010); however, only the two with oximeter tag data (G5882 and G5944) were plotted. GPS tracks of all animals were plotted using ArcGIS (version

10.2.2) to illustrate swimming locations and behaviors were similar among all of the seals and similar to previous studies. For G5882, only 14 dives were recorded with photodiode current. The slope (± SE) of the temporal change in each individual LED and the ratio of red and NIR (red:NIR) absorbance and was compared (using R, version 2.15.2). A $P < 0.05$ was used to determine significance, while a $P < 0.01$ was considered highly significant. We defined the "baseline current" as a 10 min period of the LED current reading prior to each change in current during the seals dive (Figure 6). The "dive current" is the period of time during the dive that the LED current changed from the baseline current after initial descent until the current changed back to the baseline current upon the seal's ascent.

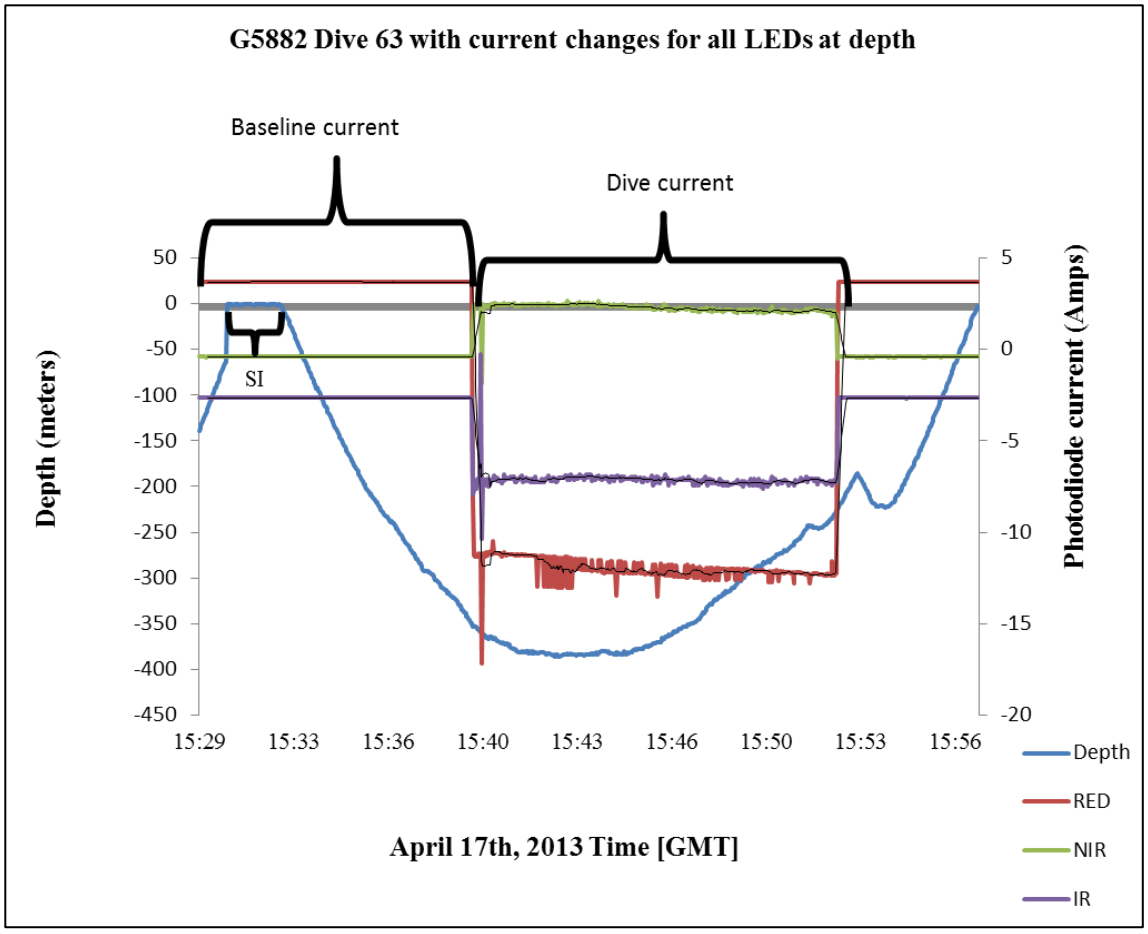

Figure 6. Dive 63 is shown here as a representative of photodiode current (Amps) for all LEDs during a dive. Depth is also shown to illustrate approximate depth (meters) when the current changed during the descent and ascent of the animal. The flat line in the current before the dive the 10 minutes of data referred to as the "baseline current." The near instantaneous changes in the current during the descent and ascent represent the beginning and end of what was referred to as "dive current." SI is the "surface interval" or time the seal spent at the surface before Dive 63. There is a sudden decrease in amps in the red LED indicating a decrease in oxygenation and a sudden increase in the NIR LED indicating a reduction in blood flow. Note also that the time the current reads as baseline exceeds the SI duration.

3. RESULTS

3.1 Practiced implantation in carcasses

Findings from practicing in the carcasses resulted in the above mentioned surgical protocol. For example, once the sensor had been pushed through the muscle fascia, we learned that turning the custom catheter at a 45° angle would allow the sensor to lie parallel within the muscle fibers instead of perpendicular to them. The idea was to eliminate as much trauma to the tissue as possible. When the sensor and surrounding tissue was excised in the carcasses, there was no indication that it had migrated within in the muscle, even when we manipulated the back end of the animal to simulate swimming movements.

3.2 Calibration of the sensor/data logger

Due to a delay in the delivery of the data loggers, we were not able to calibrate the LED and photodetectors in blood at varying saturation levels before the translocation experiments. However, before implantation we confirmed that the LEDs were working properly and that the photodetector recorded light of varying intensity. When the data loggers returned from the translocation they had been damaged and were sent back to be repaired. Once returned, calibrations in tonometered blood were attempted but no reliable relationship could be obtained. Unfortunately, we were unable to calibrate the data logger and so the results given are in Amps (A) of the photodiode current.

3.3 Field season of implantation, deployment, and data collection

3.3.1 Post-surgical observations (inflammation, infection, and swim behavior)

Findings from implantation resulted in relatively little inflammation and no signs of infection in all 5 of the implanted seals. In all of the seals, muscle placement of the sensor was confirmed and in no case was the peritoneum compromised. There were no changes observed in swim behavior from pre-surgery to post-surgical implantation of the sensor and attachment of the data logger. Following removal of the tags, the site incision/removal site was observed for any trauma. In no seal did we observe any signs of infection, or inflammation.

3.3.2 Findings from the sensor and data logger

Return to the rookery for all translocated seals took 2-7days (Table 5). GPS tracks for all animals are in Figure 7. Dive profiles for both seals with oximeter data are summarized in Table 6. Data recovered from the data logger of G5944 resulted in little over 3 hours of diving and with no data from the photosensor (Figure 8). Eighteen hours of data were recovered from G5882 and the dive record looked very similar during the first 3 hours as compared with G5944 (Figure. 8 vs Figure 9). In G5882 changes in LED absorption occurred when G5882 began diving > 350 m approximately 12 hours after the seal was released. Oximetry data was collected for 14 dives out of a total of 77 dives recorded.

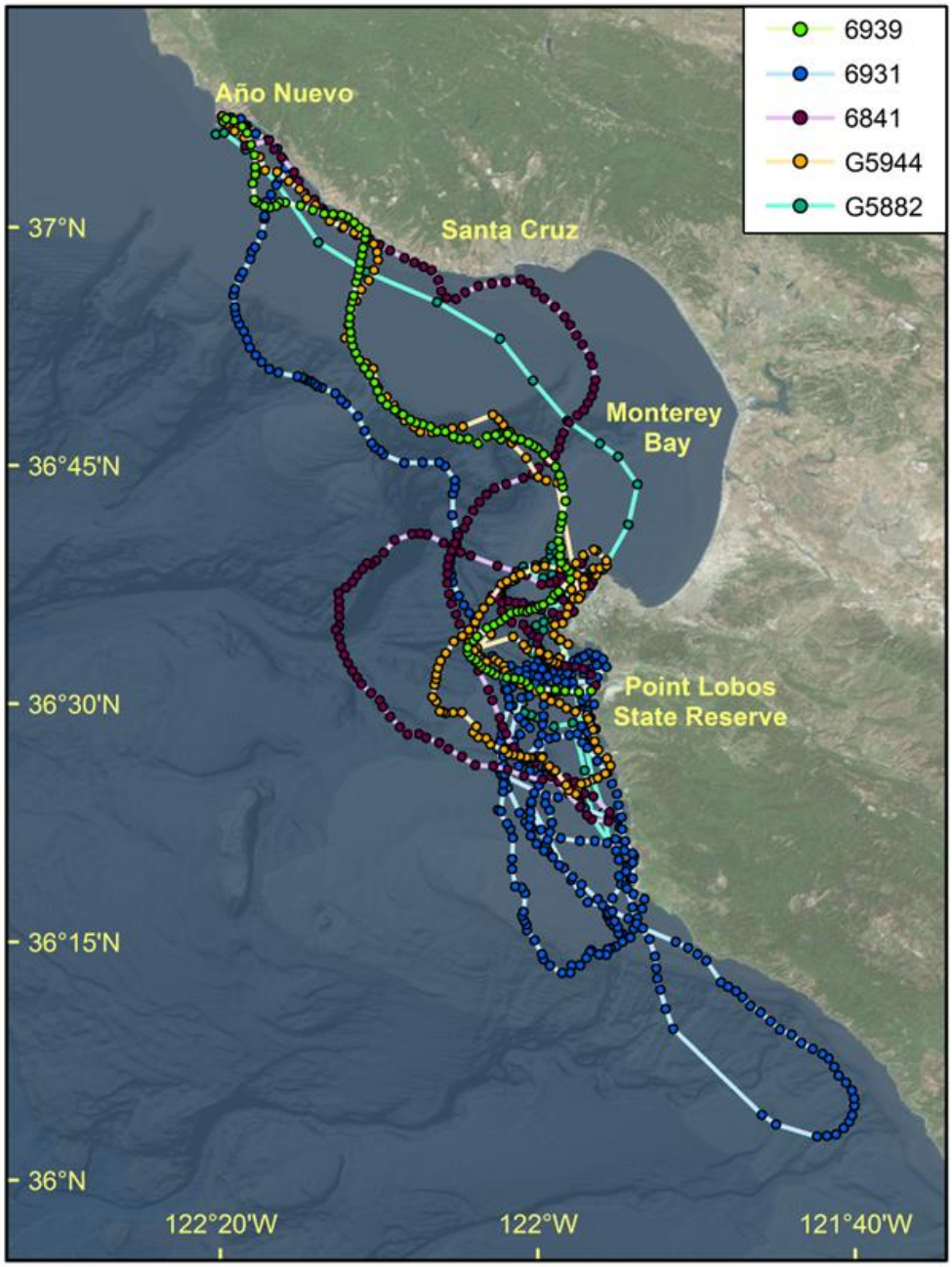

Figure 7. GPS tracks of all 5 Northern elephant seals deployed with oximeter logger. Seals 6939, 6931, and 6841 deployed with the logger implanted, but had no open ocean oximeter data logged. Seals G5944 and G5882 with open ocean data logged. The GPS tracks of all 5 animals overlaid illustrates all the animals swam around the same areas. Note 6931 came back on shore a number of times prior to swimming back across Monterey Bay.

Table 6. Data retrieved from oximeter data logger. Dive profiles recorded for both seals (G5944 and G5882) equipped with the oxygen sensor and data logger and then translocated across Monterey Bay, California.

Seal ID	Dive Record				Dive depth (m)		Dive duration (min:sec)		Surface Interval (min:sec)	
	No. hrs recorded	No. of dives	No. of dives/hr	% time at surface	Max	Mean ± SD	Max	Mean ± SD	Max	Mean ± SD
G5944	3:06:58	24	8	14.1	94.53	48.1 ± 32.3	13:14	6:42 ±3:06	8:35	1:06 ± 1:39
G5882	18:17:37	77	4.2	12.2	419.7	146.1 ± 141.0	25:00	12:31 ± 6:48	5:00	1:45 ±0:46

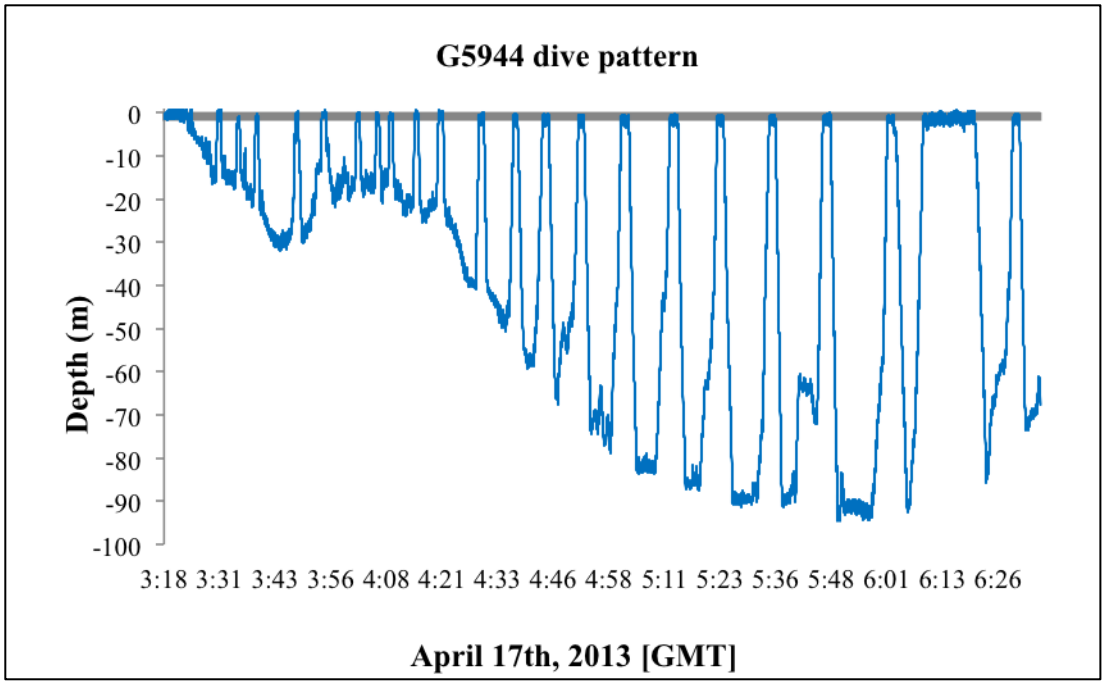

Figure 8. Dive depth (meters) and duration (minutes and seconds) recorded with oximeter data logger for seal G5944 for ~ 3 hrs on April 17, 2013, when translocated across Monterey Bay, California.

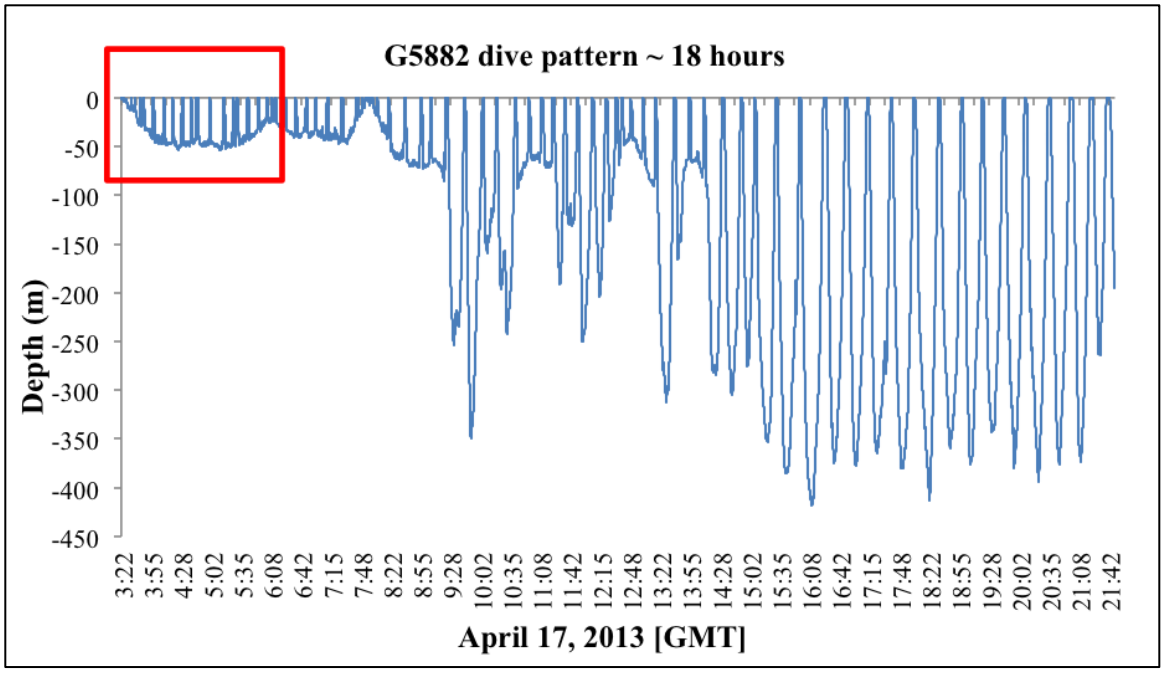

Figure 9. Dive depth (meters) and duration (minutes and seconds) recorded with oximeter data logger for seal G5882 for entire ~18 hours recorded on April 17, 2013 when translocated across Monterey Bay, California. The red rectangle highlights the first 3 hours for comparison to G5944 in Figure 8.

The profile for G5944 with 3 hours of data had a mean depth of 48.1 ± 32.3 m for a total of 24 dives (Table 6, Figure 8). In the 3 hrs of diving, G5944 spent 2 hrs and 40 min diving with only 26 minutes at the surface. Mean dive time was 6 min and 42 s ± 3 min 6 s while the mean surface interval was 1 min 6 s ± 1 min 39 s with the longest surface interval being 8 min and 35 s (46 s ± 27 s when 8 min 35 s surface interval is excluded from analysis). The longest dive duration for G5944 was 13 min 14 s.

Seal G56882 had a similar dive pattern as compared with G5944 during the first 3 hrs of diving. There were 21 dives within the first 3 hours with a mean depth of 39.4 ± 12.9 m (Table 6). Maximum depth for G5882 during the first 3 hours was 54.4 m. The mean dive duration was 7 min 45 s ± 3 min 26 s and mean surface time was 1 min 20 s ± 21 s. The maximum dive lasted 14 min and 15 s while the longest surface interval was 1 min and 45 s (Figure 9).

For the 77 dives from the oximeter logger for G5882, the mean depth was 146.14 ± 16.10 m with the shallowest dive at 2.56 m and the deepest dive (Dive 64) at 419.7 m (Figure 9). The seal spent a little over 16 hours diving and only a little over 2 hours at the surface. Mean dive duration was 12 min 31 s ± 6 min and 48 s. The minimum time spent diving was 15 s and the maximum dive was 25 minutes. The mean surface interval lasted 1 min 45 s ± 46 s with a minimum of 30 seconds and a maximum of 5 minutes.

All seals were equipped with GPS tags with ARGOS satellite uplink and had similar dive patterns to one another and to previous studies (Le Boeuf et al., 1986; Le Boeuf et al., 1988; Andrews et al., 1997). Seals 6931, 6939, and 6841 did not have data from the oximeter logger, but all seals were equipped with the GPS/ARGOS loggers to give the seals positions and dive data. The GPS tracks for each animal are in Figure 7. All 5 seals had mean dive duration of between 13 and 16 minutes with a mean surface interval of 2-3 minutes and their dives are summarized in Table 7. The mean surface interval included extended times at surface when the seals hauled back out at Pt. Lobos prior to crossing Monterey Bay. All 5 seals dive patterns are illustrated in Figures 10-14.

Table 7. The dive profiles of all seals from the GPS loggers with ARGOS satellite uplink once translocated to Pt. Lobos, CA as they swam back across Monterey Bay, CA to Año Nuevo, CA. Mean descent (D) rate, mean ascent (A) rate, mean and max surface interval (SI) are included.

Seal ID	# Days	# Dives	Mean dive depth ± SE (m)	Max depth (m)	Mean dive duration ± SE (s)	Mean D rate ± SE (m/s)	Mean A rate ± SE (m/s)	Mean SI ± SE (s)	Max SI (s)	Dives /day
6931	8	601	200 ± 153	711	866 ± 355	0.90 ± .018	0.83 ± 0.20	106 ± 201	3742	75
6939	3	154	158 ± 123	494	964 ± 352	0.66 ± 0.12	0.73 ± 0.15	143 ± 373	4654	51
6841	4	282	181 ± 167	566	795 ± 417	0.75 ± 0.21	0.86 ± 0.28	185 ± 489	4816	71
G5944	3	196	175 ± 137	457	836 ± 405	0.76 ± 0.17	0.80 ± 0.18	100 ± 102	1254	65
G5882	6	472	191 ± 170	681	883 ± 411	0.78 ± 0.21	0.76 ± 0.24	150 ± 373	4890	79

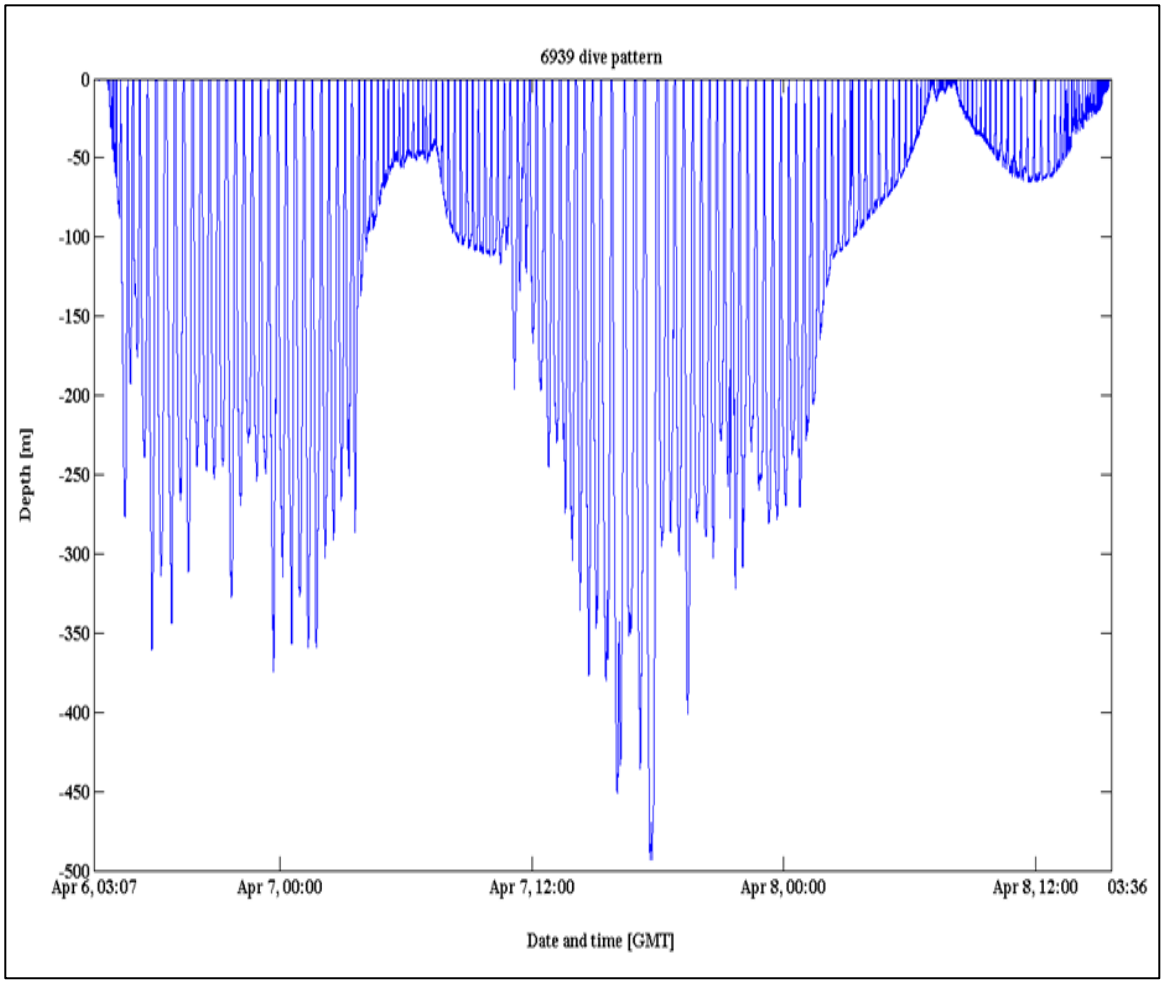

Figure 10. The dive pattern for seal 6939 as it swam from Pt. Lobos, CA back to Año Nueveo, CA across Monterey Bay. The seal was equipped with the novel oximeter sensor and logger along with GPS tagged with ARGOS satellite uplink to record muscle oxygen saturation, location, and dive data, respectively.

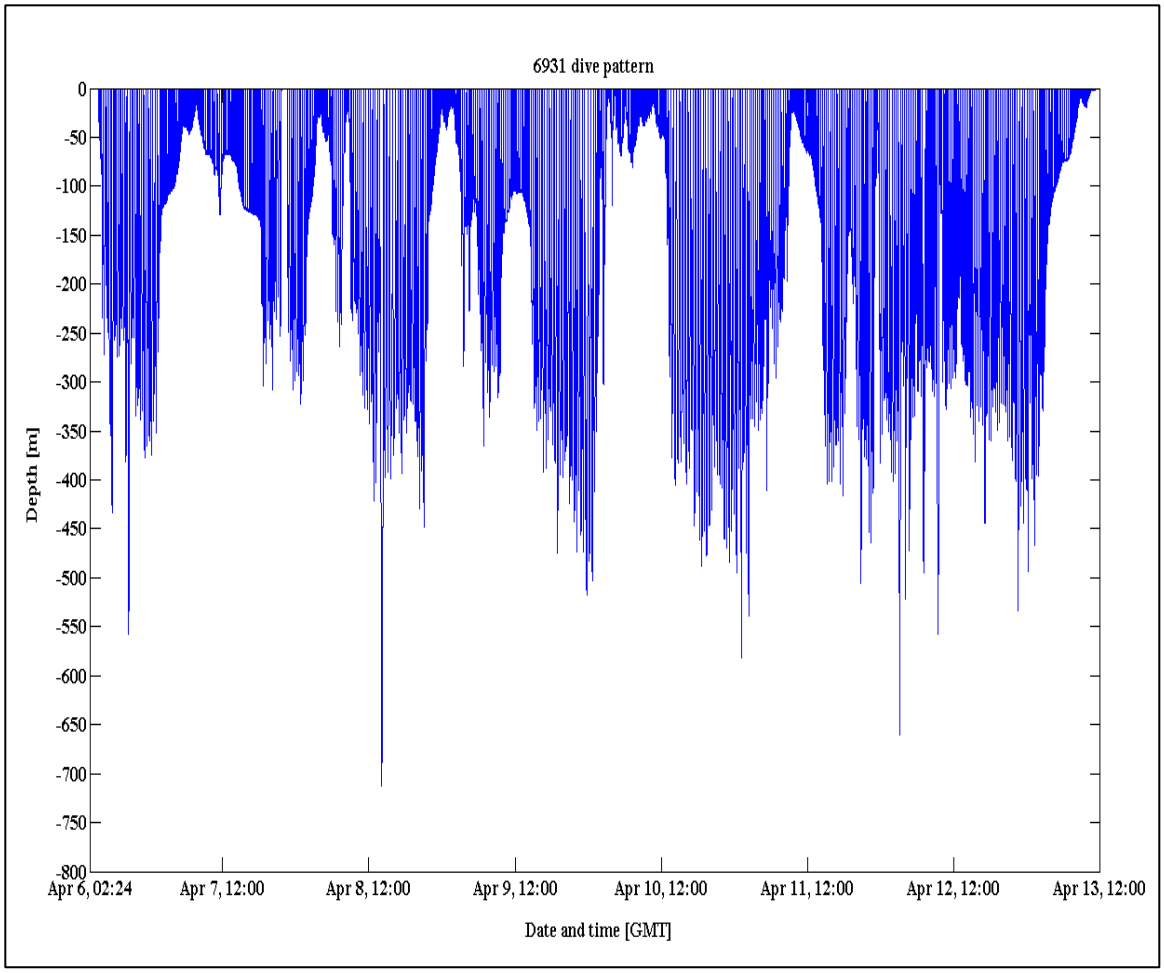

Figure 11. The dive pattern for seal 6931 as it swam from Pt. Lobos, CA back to Año Nueveo, CA across Monterey Bay. The seal was equipped with the novel oximeter sensor and logger along with GPS tagged with ARGOS satellite uplink to record muscle oxygen saturation, location, and dive data, respectively.

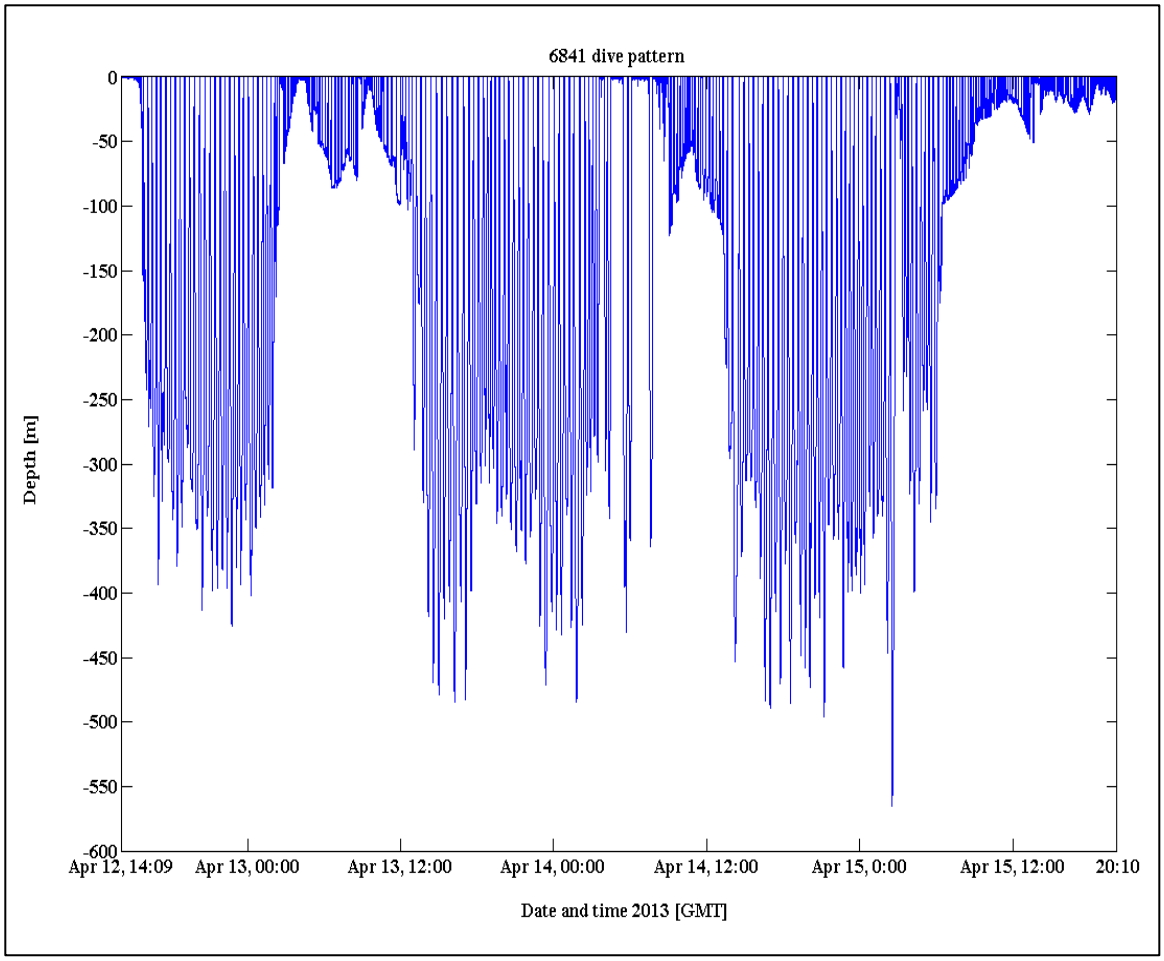

Figure 12. The dive pattern for seal 6841 as it swam from Pt. Lobos, CA back to Año Nueveo, CA across Monterey Bay. The seal was equipped with the novel oximeter sensor and logger along with GPS tagged with ARGOS satellite uplink to record muscle oxygen saturation, location, and dive data, respectively.

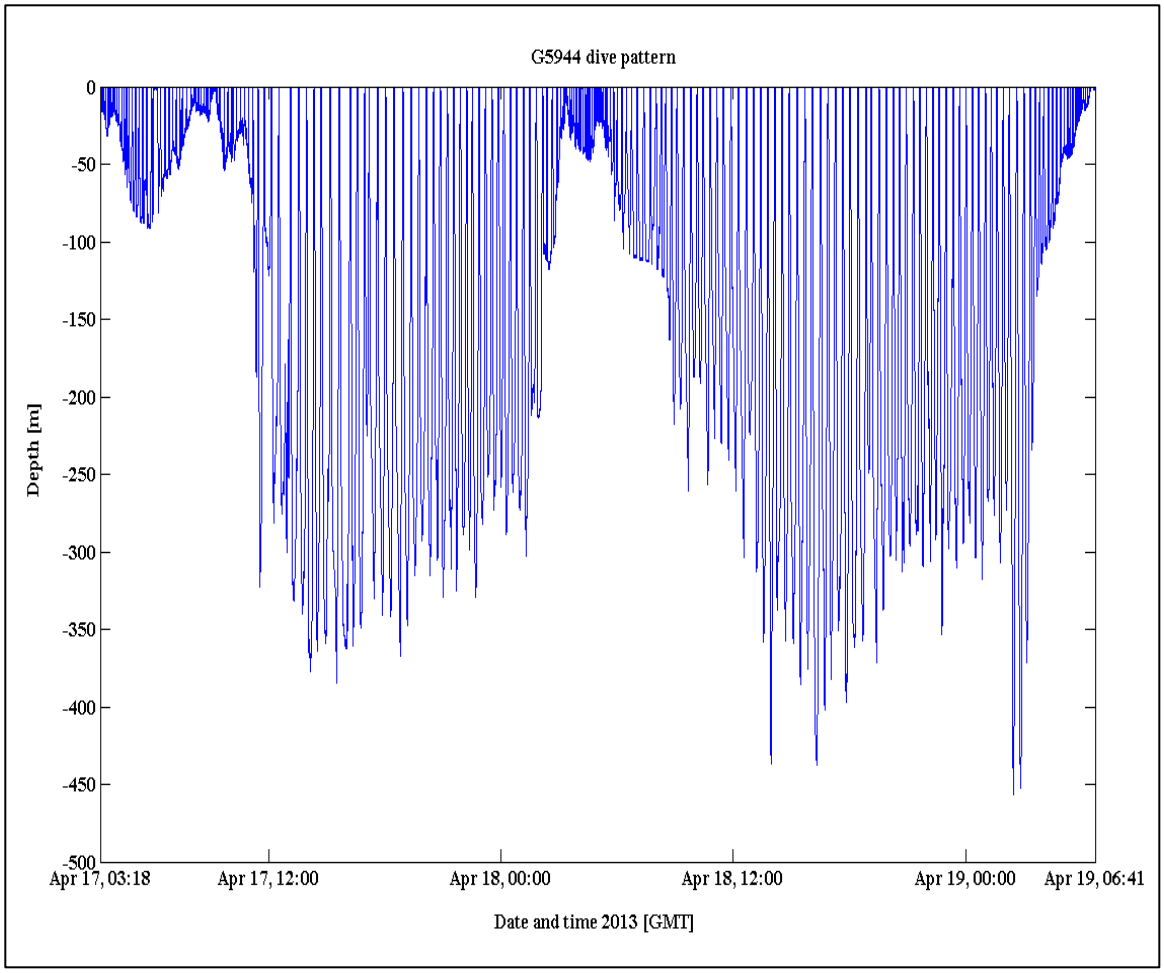

Figure 13. The dive pattern for seal G5944 as it swam from Pt. Lobos, CA back to Año Nueveo, CA across Monterey Bay. The seal was equipped with the novel oximeter sensor and logger along with GPS tagged with ARGOS satellite uplink to record muscle oxygen saturation, location, and dive data, respectively.

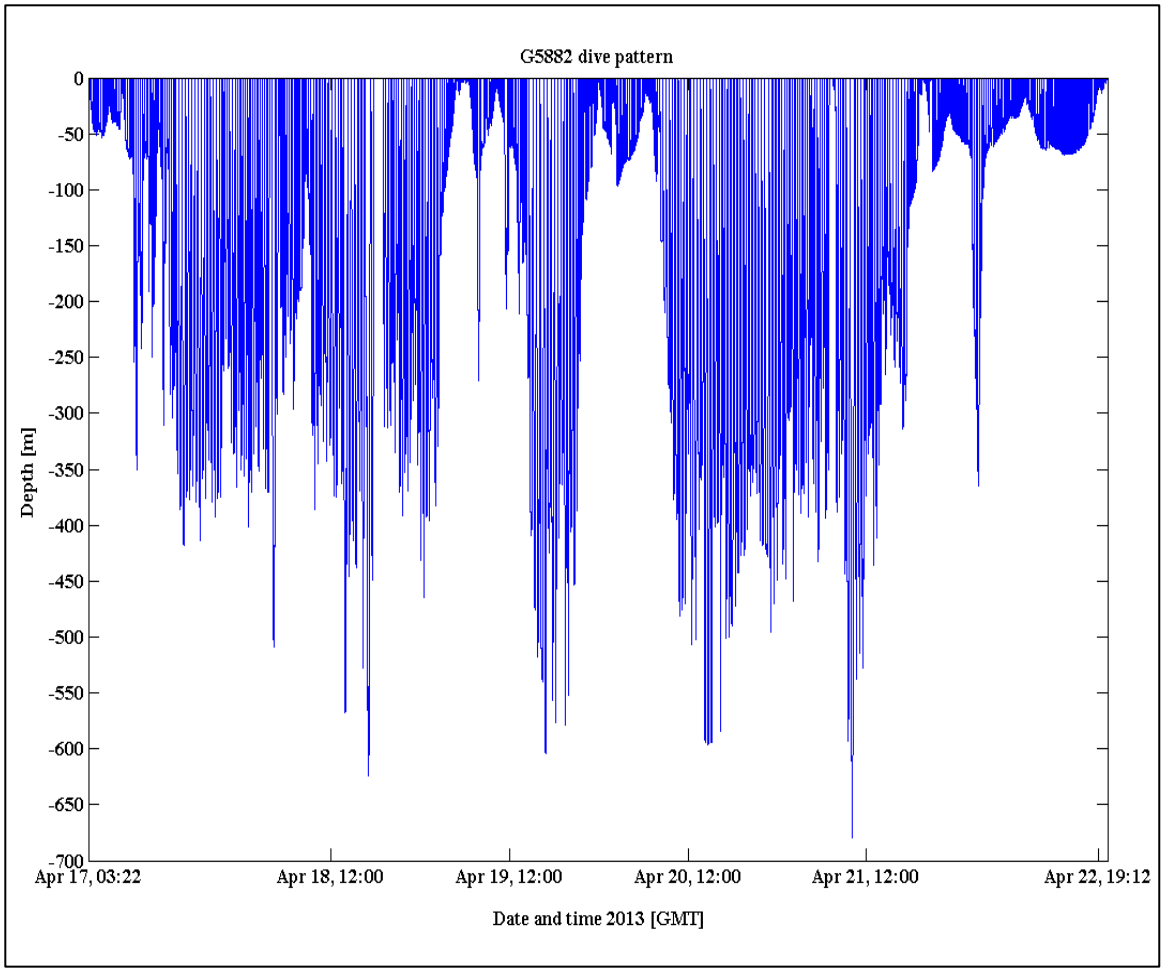

Figure 14. The dive pattern for seal G5882 as it swam from Pt. Lobos, CA back to Año Nuevo, CA across Monterey Bay. The seal was equipped with the novel oximeter sensor and logger along with GPS tagged with ARGOS satellite uplink to record muscle oxygen saturation, location, and dive data, respectively.

3.3.3 Oximetry data for 14 dives

An example of the alteration in muscle O_2 saturation can be seen in the changes in photodetector absorbance at depth in all 3 LEDs for Dive 63 is represented in Figure 6 and also illustrates the difference in "baseline current" from "dive current". For dives 63-76, the slope of the baseline current was compared to the slope of the dive current. The baseline current was not different from 0, therefore was considered to be 0. The red (660 nm) LED and the NIR (810 nm) LED all had a significant decrease in current for all dive currents as compared with the baseline current ($p < 0.01$, Tables 7 and 8), with the exception of Dive 70 in the red ($p = 0.13$, Table 7). The red:NIR ratio also had a significant decrease in current for all of the dives compared to the baseline ($p < 0.01$, Table 9). For the IR (940 nm) LED, only 9 out of the 14 dives were significantly different in the baseline current compared with the dive LED current (see p-values in Table 9).

Table 8. Red LED photodiode current (Amps). In G5882, the photodiode response for each LED in dives 63-76 were analyzed by comparing each LEDs baseline slope of 0 to the slope of each LED photodiode response during the dive ("dive current"). P-values are summarized and dives with non-significance are indicated with an asterisk. For the red LED, the parameter estimates and the standard errors (SE) for dive current are also summarized. In addition, the depth is at which the photodiode current (Amps) suddenly changed is shown during each dive when the animal was descending in the water column (D depth) and again when the animal ascended to the surface (A depth). The time (minutes and seconds) into the dive (when the seal left the surface) when the LED current changed from the baseline to "dive current" during the descent (D time) and the amount of time before the animal resurfaced when the dive current changed back to the baseline upon ascent (A time).

Dive	**Dive current slope ± SE**	**p-value**	**D depth (m)**	**D time (min:sec)**	**A depth (m)**	**A time (min:sec)**
63	$-1.31\times10^{-3} \pm 7.62\times10^{-5}$	$<2\times10^{-16}$	351	05:43	227	05:02
64	$-2.11\times10^{-3} \pm 6.85\times10^{-5}$	$<2\times10^{-16}$	289	04:20	213	03:17
65	$-2.08\times10^{-3} \pm 1.09\times10^{-4}$	$<2\times10^{-16}$	347	07:10	220	04:30
66	$-2.07\times10^{-3} \pm 1.32\times10^{-4}$	$<2\times10^{-16}$	348	06:32	218	04:26
67	$-1.26\times10^{-3} \pm 5.81\times10^{-5}$	$<2\times10^{-16}$	313	05:34	217	03:24
68	$-2.06\times10^{-3} \pm 1.41\times10^{-5}$	$<2\times10^{-16}$	353	08:30	208	03:32
69	$-2.71\times10^{-3} \pm 1.35\times10^{-4}$	$<2\times10^{-16}$	329	09:34	205	03:25
70*	$-4.49\times10^{-4} \pm 1.88\times10^{-4}$	0.13	330	08:56	208	03:44
71	$-1.80\times10^{-3} \pm 1.27\times10^{-4}$	$<2\times10^{-16}$	302	05:02	220	04:23
72	$-1.23\times10^{-3} \pm 8.48\times10^{-5}$	$<2\times10^{-16}$	303	05:56	222	05:10
73	$-1.81\times10^{-3} \pm 1.34\times10^{-4}$	$<2\times10^{-16}$	294	06:38	220	04:34
74	$-2.03\times10^{-3} \pm 1.44\times10^{-4}$	$<2\times10^{-16}$	284	07:00	213	04:06
75	$-2.44\times10^{-3} \pm 1.74\times10^{-4}$	$<2\times10^{-16}$	337	06:48	223	04:48
76	$-2.00\times10^{-3} \pm 1.41\times10^{-4}$	$<2\times10^{-16}$	339	06:06	228	04:08

Table 9. NIR LED photodiode current (Amps). In G5882, the photodiode response for each LED in dives 63-76 were analyzed by comparing each LEDs baseline slope of 0 to the slope of each LED photodiode response during the dive ("dive current"). P-values are summarized and dives with non-significance are indicated with an asterisk. For the nir LED, the parameter estimates and the standard errors (SE) for dive current are also summarized. In addition, the depth is at which the photodiode current (Amps) suddenly changed is shown during each dive when the animal was descending in the water column (D depth) and again when the animal ascended to the surface (A depth). The time (minutes and seconds) into the dive (when the seal left the surface) when the LED current changed from the baseline to "dive current" during the descent (D time) and the amount of time before the animal resurfaced when the dive current changed back to the baseline upon ascent (A time).

Dive	**Dive current slope ± SE**	**p-value**	**D depth (m)**	**D time (min:sec)**	**A depth (m)**	**A time (min:sec)**
63	$-8.96\times10^{-7} \pm 2.23\times10^{-8}$	$<2\times10^{-16}$	351	05:45	227	05:02
64	$-3.06\times10^{-4} \pm 1.09\times10^{-5}$	$<2\times10^{-16}$	297	04:29	214	03:17
65	$-6.26\times10^{-7} \pm 4.99\times10^{-8}$	$<2\times10^{-16}$	347	07:10	221	04:30
66	$-2.85\times10^{-7} \pm 5.97\times10^{-8}$	3.02×10^{-4}	348	06:44	219	04:26
67	$-1.29\times10^{-7} \pm 2.32\times10^{-8}$	0.04	313	05:34	217	03:24
68	$-5.92\times10^{-7} \pm 2.91\times10^{-8}$	$<2\times10^{-16}$	353	08:30	208	03:32
69	$-3.34\times10^{-7} \pm 4.44\times10^{-8}$	9.50×10^{-5}	329	09:36	206	03:25
70	$-1.24\times10^{-6} \pm 3.67\times10^{-8}$	$<2\times10^{-16}$	330	08:56	208	03:44
71	$-6.54\times10^{-7} \pm 2.54\times10^{-8}$	$<2\times10^{-16}$	302	05:02	221	04:23
72	$-7.49\times10^{-7} \pm 2.92\times10^{-8}$	$<2\times10^{-16}$	303	05:56	222	05:10
73	$-1.35\times10^{-6} \pm 6.01\times10^{-8}$	$<2\times10^{-16}$	294	06:38	221	04:34
74	$-1.02\times10^{-6} \pm 2.45\times10^{-8}$	$<2\times10^{-16}$	284	07:00	213	04:06
75	$-1.11\times10^{-6} \pm 6.64\times10^{-8}$	$<2\times10^{-16}$	337	06:48	223	04:48
76	$-8.49\times10^{-7} \pm 8.44\times10^{-8}$	3.88×10^{-16}	339	06:06	229	04:08

The mean signal from the red LED was 3.71 ± 0.01 A (Figure 6) at the baseline current and decreased suddenly to a mean of -11.30 ± 0.35 A during the dive at an average dive duration of 22 min 34 s ± 1 min 48 s. There was no change in the red LED baseline current (mean slope = $2.61\ 10^{-6} \pm 2.52\ 10^{-5}$ A, $P > 0.1$) while there was a significant decrease in the slope of the dive current during dives (mean slope = $-1.83\ 10^{-3} \pm 5.84\ 10^{-4}$, $P < 0.05$). The mean depth at which the NIR LED changed from baseline to dive current was 322.8 ± 24.5 m when the animal was descending while the mean depth for LED current change back to baseline current when the animal was ascending to the surface was 217.8 ± 7.1 m. The mean NIR baseline registered at -0.40 ± 0.01 A for the baseline current (Figure 6) and increased suddenly to the mean NIR dive current of 2.49 ± 0.09 A (Figure 6) with the same average depth and dive duration for the red LED. There was no change in the NIR LED baseline current (mean slope = $1.53\ 10^{-6} \pm 5.74\ 10^{-6}$ A, $P > 0.05$), however there was a significant decrease in the slope of dive current during dives (mean slope = $-2.30\ 10^{-05} \pm 8.17\ 10^{-05}$ A, $P < 0.05$). The mean depth for LED change from baseline to dive current during descent was 323.4 ± 23.9 m and during the ascent was 217.8 ± 7.1 m. Although there was an overall continuous decrease in the slope of the dive current, in some of the dives (67, 69, and 75) there were sudden increases in the NIR current.

For Dives 63-76, the red LED current was divided by the NIR LED current providing a ratio for desaturation while taking into account changes in blood flow. The trends in the red:NIR ratio followed the same patterns for the two LEDs with overall desaturation with what looked like occasional blood flow back into the muscle. For the

red:NIR ratio, the mean slopes for the baseline current were -4.79 10^{-05} ± 3.59 10^{-04} and then decreased for the dive current at mean slope -1.73 10^{-03} ± 5.55 10^{-04} (Table 10).

Table 10.The red:NIR ratio of photodiode current (Amps). In G5882, the dive current slope of the red LED was divided by the dive current slope of the NIR LED to get a ratio of the two for dives 63-76. The red:NIR ratio was analyzed by comparing each dive baseline of 0 to that of the red:NIR ratio each LED photodiode response during the dive ("dive current"). P-values are summarized. For the red:NIR ratio, the parameter estimates and the standard errors (SE) for dive current are also summarized.

Dive	Dive current slope ± SE	p-value
63	$-2.19\times10^{-3} \pm 4.69\times10^{-5}$	$<2\times10^{-16}$
64	$-1.62\times10^{-3} \pm 3.70\times10^{-5}$	$<2\times10^{-16}$
65	$-1.65\times10^{-3} \pm 8.03\times10^{-5}$	$<2\times10^{-16}$
66	$-5.04\times10^{-4} \pm 9.66\times10^{-5}$	0.00
67	$-8.76\times10^{-4} \pm 5.21\times10^{-5}$	1.16×10^{-6}
68	$-1.76\times10^{-3} \pm 8.33\times10^{-5}$	$< 2\times10^{-16}$
69	$-1.68\times10^{-3} \pm 8.63\times10^{-5}$	$< 2\times10^{-16}$
70	$-1.69\times10^{-3} \pm 8.96\times10^{-5}$	$< 2\times10^{-16}$
71	$-1.67\times10^{-3} \pm 6.89\times10^{-5}$	$< 2\times10^{-16}$
72	$-1.44\times10^{-3} \pm 5.83\times10^{-5}$	$< 2\times10^{-16}$
73	$-2.54\times10^{-3} \pm 1.02\times10^{-4}$	$< 2\times10^{-16}$
74	$-2.46\times10^{-3} \pm 7.46\times10^{-5}$	$< 2\times10^{-16}$
75	$-2.23\times10^{-3} \pm 1.14\times10^{-4}$	$<2\times10^{-16}$
76	$-1.91\times10^{-3} \pm 1.13\times10^{-4}$	$<2\times10^{-16}$

For the IR LED, five of the dives had dive currents during the dive that were not significantly different from the baseline current (Dives 65, 68, 71, 72, and 76; P > 0.05, Table 11). The mean IR baseline current registered at -2.65 ± 0.01 A (Figure 6) and decreased suddenly to -6.96 ± 0.16 A (Figure 6) during the dive at the same depths and

durations as the prior two LEDs. Also, mean depth for LED changed from baseline to dive during descent and ascent were exactly the same as for the IR LED as for the NIR LED.

Table 11. IR LED photodiode current (Amps). In G5882, the photodiode response for each LED in dives 63-76 were analyzed by comparing each LEDs baseline slope of 0 to the slope of each LED photodiode response during the dive ("dive current"). P-values are summarized and dives with non-significance are indicated with an asterisk. For the IR LED, the parameter estimates and the standard errors (SE) for dive current are also summarized. In addition, the depth is at which the photodiode current (Amps) suddenly changed is shown during each dive when the animal was descending in the water column (D depth) and again when the animal ascended to the surface (A depth). The time (minutes and seconds) into the dive (when the seal left the surface) when the LED current changed from the baseline to "dive current" during the descent (D time) and the amount of time before the animal resurfaced when the dive current changed back to the baseline upon ascent (A time).

Dive	Dive current slope ± SE	p-value	D depth (m)	D time (min:sec)	A depth (m)	A time (min:sec)
63	$-3.32\times10^{-4} \pm 2.54\times10^{-5}$	4.44×10^{-12}	350	05:43	227	05:02
64	$9.85\times10^{-5} \pm 1.56\times10^{-5}$	0.03	297	04:29	214	03:17
65*	$9.52\times10^{-5} \pm 3.57\times10^{-5}$	0.06	347	07:10	221	04:30
66	$6.48\times10^{-4} \pm 5.07\times10^{-5}$	$<2\times10^{-16}$	348	06:32	219	04:26
67	$2.35\times10^{-4} \pm 2.06\times10^{-5}$	2.90×10^{-7}	313	05:34	217	03:24
68*	$2.45\times10^{-5} \pm 2.73\times10^{-5}$	0.4	353	08:30	208	03:32
69	$4.69\times10^{-4} \pm 6.66\times10^{-5}$	8.69×10^{-6}	329	09:36	206	03:25
70	$-6.51\times10^{-4} \pm 3.63\times10^{-5}$	$<2\times10^{-16}$	330	08:56	208	03:44
71*	$-1.20\times10^{-5} \pm 4.09\times10^{-5}$	0.56	302	05:02	221	04:23
72*	$-2.20\times10^{-5} \pm 4.03\times10^{-5}$	0.55	303	05:56	222	05:10
73	$-2.20\times10^{-4} \pm 5.54\times10^{-5}$	0.01	294	06:38	221	04:34
74	$-3.45\times10^{-4} \pm 3.15\times10^{-5}$	1.81×10^{-10}	284	07:00	213	04:06
75	$-2.67\times10^{-4} \pm 4.39\times10^{-5}$	5.14×10^{-6}	337	06:48	223	04:48
76*	$3.98\times10^{-5} \pm 5.35\times10^{-5}$	0.53	339	06:06	229	04:08

4. DISCUSSION

Assuming the logger was working properly, findings from the sensor/data logger indicated a decrease in blood flow to the muscle at approximately the same depths as the animal descended in the water column (Tables 8, 9, and 10). There was decrease in blood flow to the swimming muscle as indicated by the change in current in the NIR LED during the dive and desaturation at the muscle as indicated by small current changes in the red LED. Unfortunately, all attempts to calibrate the data logger were unsuccessful and the data logger only provided oximeter data from one of the five seals implanted. Dive profiles of all translocated seals followed the same patterns and characteristics as found in previous studies (Le Boeuf et al., 1986; Le Boeuf et al., 1988; Andrews et al., 1997). The developed surgical protocol was determined to be aseptic and minimally invasive as no signs of inflammation or infection were found in any of the seals implanted. Although the data was limited, we learned how to implant an oximeter sensor into the major swimming muscle of the Northern elephant seal with minimal trauma and with no effects to their natural swimming and behavior. Our findings lend significant insight into the potential for further exploration with a few adjustments to the logger.

4.1 Calibration for the sensor/data logger

The objective with this study was to measure muscle O_2 saturation during diving in the elephant seal. However, as we were unable to properly calibrate the sensors against known Hb saturation only trends and qualitative data are reported and discussed later.

There may be a couple of reasons for the inability to calibrate the logger. Only one of the sensors continued working intermittently following its recovery from the animal in the field. The sensor and logger were returned to the engineer for repairs. Upon return of the sensor and data logger from the engineer to our laboratory, one of the pins in from the silver wires to the electronic board was loose at the connection to the data logger and the sensor LEDs were not working. We speculate the damage occurred in the shipping process. Additional repairs were made, including soldering the loose wires, and the sensor started working again. Tests alternating placement of the sensor in the dark and exposure to light indicated that the sensor was once again working. The loose wire continued to disconnect from the electronics board. By the third attempt using isolated Hb, pins 1 and 4 were also coming loose. The loose wires may not have allowed the logger itself to work properly thereby inhibiting the ability to calibrate it. All calibration attempts were unfruitful as a linear relationship was not established from PO_2 measurements and readings from the sensor/data logger.

In fact, we tried to test the logger by immersing the sensor in a tube blackened with electrical tape and topped with a rubber stopper so as to allow no light penetration. The tube was injected with 0.1 ml of red food coloring every 60 seconds for 10 minutes while the data logger recorded changes in current from each of the 3 LEDs. The results from this small experiment were still noisy, unreliable and non-linear.

Further potential reasons for failure to calibrate the logger might be in the volumetric mixing technique. Human whole blood was used for calibration at first and then an attempt was made to calibrate it with isolated Hb. The tonometering time of 30

minutes may not have been enough to obtain fully oxygenated or deoxygenated whole blood samples as relatively little color differentiation was observed in whole blood samples compared to isolated Hb. Any more than 30 minutes of tonometering of the samples resulted in clumping of the blood. In addition, if saturation values of 0% and 100% are not achieved, subsequent mixing would result a solution with unknown saturation. However, similar non-linear results were obtained when we attempted to calibrate with isolated Hb indicating that there problems beyond the mixing technique. While there were issues getting solutions of the appropriate saturation, it is just as likely that the oximeter logger does not work properly.

4.2 Post-surgical observations

Relatively little inflammation and no signs of infection were recorded in all 5 of the implanted seals following surgery, pool recovery, and translocation recovery. In fact, two seals, 6931 and 6939 (the first two seals implanted) were found again a week after the probes and data loggers had been removed at Año Nuevo. There were still no signs of inflammation and infection at the implantation sites for these two seals indicating that the implantation had no residual or harmful effects.

During recovery of the data logger from the first two seals, 6931 and 6939, it was discovered that the battery life was considerably shorter than the expected 5 day recording time; however, these first two seals provided invaluable observational data while recovering in the pool for ~18 hours. For the third implanted seal (G6841) the

magnetic on/off switch being was activated before release, which drained the data logger battery.

We rectified this for the 4th and 5th animals (G5944 and G5882) and made sure that the data logger was not activated before release into the bay. Confirmation via ultrasound of stable embedding of the provided sensor into a major swimming muscle, collection of data from the logger, and observation of the 5 seals instrumented lead us to conclude that the updated sensor and data loggers could be successfully deployed in a second field season with improvements made to the instrumentation. We conclude that lack of infection and no changes in swim behavior are indicative of good surgical implantation technique.

4.3 Findings from the sensor and data logger

4.3.1 Dive profiles

When comparing the first 3 hours of G5882 to the entire 3 hour dive of G5944, both animals began with relatively shallow dives slowly increasing the depth and duration of the dives. Both animals dove to similar depths and had similar dive durations, however, G5944 had a surface interval that lasted ~8.5 minutes while G5882 did not. The dive profiles of G5944 and G5882 were similar to one another during the first 3 hours after being released. Once G5882 swam beyond the continental shelf, the dives fit in with the characteristic dives observed in previous studies with dives between 20-30 minutes with ~2 minutes of surface time in between (Le Boeuf et al., 1986; Le Boeuf et al., 1988; Andrews et al., 1997).

4.3.2 Photodiode current for LEDs

We were able to gather oximetry data for 14 dives of the 77 for seal G5882. What caused the sensors to suddenly come on at right before Dive 63 is unknown although it did occur after the seal reached a depth of > 350 m. Again, this could be due to the fact that pressure had an effect on the data logger as not all of the loggers were repeatedly pressure tested and the pressure sensor may have come loose.

Oxygenated blood absorbs less light at the red wavelength (600nm) as compared with deoxygenated blood (Fig. 1 and 2 in Ch1). Thus, as the tissue becomes deoxygenated the absorbance of light by the tissue should increase. The photodetector detects light levels and increasing light increases the current (Amperes) recorded. Consequently, if the tissue absorbs more light then less light will reach the photodetecter thereby decreasing the current signal. Thus, at 660nm we would expect the current to decrease as the tissue goes from oxygenated to deoxygenated. The opposite is true for the IR signal (940 nm), while the NIR depends on the Hb concentration and should decrease with increasing [Hb].

The red LED indicated oxygenated blood/tissue during the baselines based on the relatively higher current than when the seal was diving. At depth, the current decreased quickly to the lower signal, which indicated a change in oxygenation. The decreasing mean slope of the dive current indicated that as the dive progressed, the tissue was deoxygenating. In most of the dives, there were slight increases and decreases in the current that looked like the signal may have either been getting less noisy or that there

were changes in oxygenation during the dive at the tissue. Good examples of this were found in dives 64, 67-71, and 74. The dive current slope for dive 70 showed increases and decreases as well which was suggestive of changes in blood flow; however it should be noted that the overall slope was not considered different statistically from the baseline ($p = 0.13$). The non-significance may be due to the fact that the increases and decreases looked somewhat cyclical in nature, cancelling each other out so that the overall slope was deemed not significantly different from the baseline. After the animal began to ascend back to the surface the LED quickly changed back to the baseline current long before the animal reached the surface.

The NIR LED increased from the mean baseline current to a relatively higher current during the dives which indicated a quick change in blood flow to a decreased level. The change in this LED readout occurred as the animal was diving to depth. During the dives, the slope of the NIR was decreasing which indicated a decrease in blood flow during the dive even beyond the initial decrease at 323 m. However, occasionally, there were sudden increases in the NIR current indicating a brief periods of blood flow into the muscle during the dives. As the seal ascended to the surface, the LED signal nearly instantaneously changed back to the baseline just as in the RED led signal had before the animal reached the surface.

The red and NIR LEDs behaved as we expected. While the IR LED readings in the baselines also behaved as expected, the IR readings during the dives did not. As mentioned above, we expected to see a mirror-image of the red LED signal but on a smaller scale in variance. The mean baseline readings for the IR LED registered at -2.65

A as opposed to the 3.71 reading from the red LED. The IR LED unexpectedly dropped to -6.97 A. This drop in IR current was unexpected as the red LED also dropped when changing from baseline current to dive current. During the dives, the IR LED indicated a decreasing slope of $-2.2\ 10^{-05}$ instead of an increase. Currently, there is no way to explain why the IR LED behaved in this way. Perhaps the LED was wired in reverse.

Lastly, the ratio between the red and NIR LED was analyzed. The ratio between these two LEDs gives us a relative index of O_2 desaturation independent of Hb concentration. There were still significant differences between the current baselines and diving current while diving (Table 10).

Although not physiological, one explanation for the sudden changes in the LED currents at approximately the same depth is that there might be a malfunction in the pressure transducer. It was later discovered that the engineer may not have repeatedly pressure tested all of the loggers and the pressure sensor may have come loose. All LEDs showed a drastic change from baseline to dive current and back at approximately the same time and depth. If this is not mechanical error, could these be the depths that the animal collapses the lung when descending and re-inflates when going back to the surface?

Assuming the logger is functioning properly in the last seal, we have tried to tease out what might have occurred physiologically. We see decreased blood flow in the muscle as indicated by the NIR and a slow desaturation of the muscle during the dives as indicated by the small change in current in the red (Figure 6). Physiologically, we speculate the blood is quickly shunted away from the swimming muscle via

vasoconstriction once the animal reaches a certain depth (~323 m) and what remains is deoxygenated until a re-perfusion to the swimming muscle upon ascension to the surface. This study did agree with Guyton et al. (1995) in that the NIR (810 nm) wavelength showed blood flow returning to baseline late in the dive. The re-perfusion during the animal's ascent may be due to the pre-surface tachycardia and vasodilation that is known to occur in this species (Thompson and Fedak, 1993; Meir et al., 2009; Ponganis et al., 2011). It also is quite possible that if the blood is shunted away to other organs like the brain and heart, what we see here is the deoxygenation of the myoglobin as myoglobin is optically similar to hemoglobin at these LED wavelengths (Williams et al., 2011).

The fact that the LED changes occur at around the same depth for each dive is that the animal may have become negatively buoyant and is gliding to depth as seen in other studies (Williams et al., 2000). As the muscle is not working as vigorously during a glide to depth, blood flood may not be as needed and is therefore shunted away from the muscle to the organs. Upon the animal's ascent, the animal may need to use the swimming muscle to actively stroke toward the surface. A re-perfusion of blood at this critical time may explain the sudden change back to baseline that we observed in this study. As we have not measured heart rate or acceleration during this study, we can only speculate these relationships. The data in the current study are similar to findings in Meir et al. (2009) where arterial and venous blood O_2 depletion data did support the idea of maintenance of muscle blood flow during descent to depth. Additionally, our findings were similar to Guyton et al. (1995) where muscle O_2 declined throughout the dive. Guyton et al. (1995) also found in 2 Weddell seals a partial resaturation of the muscle.

We may have also found evidence of a partial resaturation in these 14 dives of this particular animal as indicated by the sudden increases in the NIR current.

The red LED seemed to be much noisier than the other two LEDs. During data analysis, the red dive data appeared to possess 3 slopes within the dive current readouts. We concluded that using a single slope to represent these 3 trends was not a distortion of the data and that there appeared to be a mechanical error, or noise, in the red LED. This same distortion did not show up in the NIR or IR LED readings, albeit there was noise in each of them as well.

One explanation for the noise was that it could be muscle movement artifact; however, that conclusion was discarded as we might have seen it in the other two LEDs, but did not. Another explanation is that the photodetector of the sensor is right next to the red LED instead of on the other side of it. The signal from the red LED may be stronger due to its proximity to the photodetector.

4.4 Future work and recommendations

Findings from the field work in this study indicate the need for several improvements to the data logger before deployment in future field seasons or studies.

The location of the battery directly beneath the board was an issue. The wire placement above the electronics board made replacing the battery without damaging or pulling the wires tumultuous. Also, the battery was found to last only ~12-18 hours. Securing the battery within the housing is of utmost importance as is extending the

battery life. The quality and quantity of recordings significantly depended on battery operation.

Analyzing the data collected was difficult without a proper timestamp as there was no internal clock. One of the 3 LEDs could be sacrificed in order to draw less power (improve battery life) so that an internal clock can be added. The IR LED should be the one sacrificed as it was the one that gave us the most unreliable data. There is a strong need for this improvement. One suggestion for improvement in this area is to install a programmable clock for start and stop times.

The sensor head must also be pressure tested. This would ensure proper functioning of the sensor head at different pressures. This would allow us to determine if any shifts or "noise" in the data are real or due to malfunctions in the sensor electronics due to pressure changes.

Lastly, the current design operates with a magnetic on/off switch that is located on the external area of the logger housing. When we are ready to turn on the recorder, the magnet must be removed. This became problematic in the field when the magnet was prematurely dislodged and began collecting data and draining the battery. In addition, it can be difficult to remove from an animal combatting the researchers at every turn.

5. CONCLUSION

Although the data was limited, our findings from this first field season were important for the field of diving physiology. First, we were able to develop a surgical

technique that minimized the invasiveness of implanting a small sensor into the major swimming muscle of the Northern elephant seal. We were also able to observe the animals in the pool post-surgery, during the translocation via ARGOS, and in the field when we removed the device. There were no observable effects to their swim behavior from the oximeter sensor implantation. Furthermore, we were able to obtain data by the end of the field season from one seal out of five indicating that with a few adjustments to the data logger, a second field season may yield a larger sample size and more reliable data.

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